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Breeding resistance in strawberry cultivars for *Fusarium oxysporum* f. sp. *fragariae*

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Abstract

An alternative method of disease management is needed to combat the high incidence of Fusarium wilt of strawberry, caused by *Fusarium oxysporum* f. sp. *fragariae* (*Fof*), in Queensland and Western Australia following the phase-out of methyl bromide fumigation. The use of Fusarium wilt resistant cultivars as part of an integrated disease management plan could play an important part in reducing losses to this disease.

The variations in the virulence that may exist among *Fof* strains, and the variation in susceptibility among strawberry genotypes to *Fof* strains, are important aspects in screening for resistance to ensure disease exposure and expression are adequate for assessment. As no research has been done regarding the genetic variability of Australian *Fof* strains, 25 isolates of *F. oxysporum,* obtained from the major strawberry production areas in Australia, were characterised using pathogenicity testing, vegetative compatibility groups (VCGs) based on complementation testing of mutants, and molecular techniques using translation elongation factor-1 alpha (EF-1α) and mitochondrial small subunit (mtSSU) ribosomal DNA gene analyses. Disease development response determined from pathogenicity tests showed significant variation among isolates; from these tests, four pathogenic isolates were chosen for use in cultivar evaluations and screening for *Fof* resistance. Two distinct VCGs were identified which closely corresponded to two of the ten lineages identified by partial EF-1α sequence phylogenetic analysis.

An evaluation of cultivar resistance to isolates of *Fof* showed significant differences, ranging from highly resistant to highly susceptible. Cultivar × isolate interactions performed by fitting mixed models with smoothing splines determined responses ‘over time’ as significant. The isolates from Western Australia were distinct from the Queensland isolates and were more virulent to the dominant strawberry cultivar grown in that region.

To further develop a screening technique suitable for use in the screening process for *Fof* resistance and for large scale screenings, two conidial-suspension methods (root dip and injected) as well as three conidial concentrations within the root dip, and two incubated seed-carrier (ryegrass and millet) inoculation methods were compared. Disease incidence and severity ratings were used to determine *Fof* infection and disease development. No significant differences were observed among conidial concentrations. Ryegrass inoculation proved to be a very effective method that produced higher disease rating than the other

treatments. A modified root dip method incorporating ryegrass seed was subsequently used in the resistance screening.

Experiments were performed to identify individual breeding values and determine the inheritance of the resistance trait in strawberry to provide sufficient knowledge to allow for future development of *Fof* resistant strawberry cultivars. Best performing genotypes were determined using a general linear mixed model (GLMM), incorporating a pedigree. Variation in disease response of the screened population implies a quantitative effect. The estimate of the narrow sense heritability (0.49) suggests that the screened population would be responsive to phenotypic recurrent selection. While this study is based on the breeding values from one breeding population, information gained suggests *Fof* resistance can be incorporated into commercially suitable genetic background.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Incorporated as Chapter 6

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Author Mark Herrington	Designed experiments (15%) Edited paper (65%)

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Contributions by others to the thesis

Joanne De Faveri contributed to the analysis and interpretation of the research data and critically revised sections of the statistical section of the paper (Chapter 5).
Mark Herrington contributed significantly in the conception and design of parent crossings to be used in screenings (Chapter 5)
Elizabeth Czislowski contributed to technical work and analysis and interpretation of the genetic studies (Chapter 2).

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Keywords

Fusarium wilt, strawberry, heritability, linear mixed model, vegetative compatibility group, translation elongation factor 1α, mitochondrial small-subunit.

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FoR code: 0604 Genetics, 50%
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List of Abbreviations

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
Avr	Avirulence
BLUPs	Best linear unbiased predictions
bp	base pairs
DNA	Deoxyribonucleic acid
EF-1α	Elongation factor 1-alpha
ETI	effector-triggered immunity
Foc	*Fusarium oxysporum* f. sp *cubense*
Fof	*Fusarium oxysporum* f. sp *fragariae*
Fol	*Fusarium oxysporum* f. sp. *lycopersici*
Foz-	*Fusarium oxysporum* f. sp. *zingiberi*
f. sp.	*forma specialis*
GLMM	General linear mixed model
HST	host-specific toxin
HR	hypersensitive response
IGS	Intergenic spacer
MAMP	microbe associated molecular patterns
MCL	Maximum composite likelihood
ML	Maximum Likelihood
MRF	Maroochy research facility
MTI	microbial-associated molecular-patterns-triggered immunity
mtSSU	Mitochondrial small subunit
nit	nitrate nonutilising
PAMPs	pathogen-associated molecular patterns
PCR	Polymerase chain reaction
Qld	Queensland
QTL	Quantitative trait loci
R	Resistance gene
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rDNA	Ribosomal DNA

SA	South Australia
SAR	systemic acquired resistance
SPDA	¼ strength potato dextrose agar, amended with 50 ppm streptomycin sulphate
VCGs	Vegetative compatibility groups
WA	Western Australia

Introduction to thesis

Strawberries are grown in the coastal regions of most Australian states, and have a farm gate value of $400 million year^{-1} (HAL, 2012). Strawberry plants are clonally propagated and mostly planted annually during March through May, depending on location, to supply markets throughout Australia. For southeast Queensland (Qld) this is from May to October, while Victoria and other southern states supply the rest of the year. These timings and locations are influenced by rainfall and the photoperiod × temperature × cultivar interactions that affect runner and fruit production. Establishment and growth is a significant investment, averaging $136,524/ha in Qld (QSIPC, 2006). Consequently, the subsequent death of established plants from soil-borne diseases carries a substantial economic penalty so that fruit producers try to minimize the risk of such losses.

For much of the past 40 years, strawberry runner and fruit growers in Australia have relied heavily on pre-plant fumigation, using methyl bromide/chloropicrin mixtures for the effective control of soil-borne diseases. Because methyl bromide was so effective and economically competitive, growers and breeders placed little importance on developing cultivars resistant to soil-borne diseases affecting strawberry. As a consequence Australia has no cultivars available that were bred specifically for resistance to any of the major soil-borne diseases.

In Australia, the complete withdrawal of methyl bromide (under the 'Montreal protocol') occurred in 2005, resulting in the use of alternative fumigants that are less effective than methyl bromide (Hutton and Gomez, 2010). Additionally, as a means to reduce cost and resource requirements, practices such as replanting into used plastic mulch and ratooning (i.e., carrying plants over into a second production season) have increased. Associated with these changes, the incidence of and economic loss resulting from fungal soil-borne pathogens have increased. Fusarium wilt of strawberry caused by the soil-borne disease *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) (Winks and Williams) has caused large losses to strawberry production worldwide. In Australia outbreaks have been particularly apparent in Qld and Western Australia (Winks and Williams, 1965; Hutton *et al.*, 2004; Golzar *et al.*, 2007). However, pathogenicity, and the genetic diversity of *Fof* isolates associated with these outbreaks in Australia are largely unknown.

Due to the economic loss caused by soil-borne diseases such as Fusarium wilt (Phillips and Golzar, 2008) and the push for usage of so called softer chemicals (Duniway, 2002), strawberry growers and breeders have had to look at other control management

strategies. Breeding cultivars with resistance to Fusarium wilt is therefore of great importance to the success and sustainability of production and could provide a long term alternative to the use of fumigants (Hutton and Gomez, 2010). Knowledge of pathogen virulence is an important aspect for resistance breeding and selection, enabling strawberry genotypes to be tested against a wide range of pathotypes.

Accordingly, the primary objective for the study was to provide sufficient knowledge to allow for more precise predictions and interpretations of the genetics of the pathogen and host. This will facilitate the future development of *Fof* resistant strawberry cultivars, offering growers a sustainable long-term approach to Fusarium wilt control. Therefore, this study aimed to: (i) assess the variability of *F. oxysporum* species obtained from diseased strawberry, (ii) examine their pathogenicity on strawberry, allowing for selection of isolates for use in plant resistance screening; (iii) examine and compare methods of inoculation concentration and delivery to determine the most effective screening protocol; and (iv) evaluate the relative susceptibility of strawberry genotypes to *Fof*, and determine breeding values to assist breeders improve the effectiveness of genomic selection of best parents for future crosses.

1.0 Chapter 1 Literature review and aims of this study

1.1 The strawberry

The strawberry is an herbaceous perennial belonging to the Rosaceae family, and can be found growing in most arable areas of both the Southern and Northern Hemisphere. The cultivated strawberry, *Fragaria* × *ananassa* Duchesne ex Rosier, is an octoploid (2*n*=8*x*=56); derived from a cross between *F. virginiana* from eastern North America and *F. chiloensis* from Chile and Argentina (Darrow, 1966). *Fragaria* × *ananassa* dominates the commercial production of strawberries and is considered economically the most important soft fruit worldwide (Hummer and Janet, 2009).

1.1.2 The Australian Strawberry Industry

Strawberries are a popular fruit in Australia (Cooke *et al.,* 2009) and approximately 30,000 tonnes of strawberries are produced annually (FAOStat, 2010). Fruit production is primarily concentrated in the sub-tropical and temperate coastal regions of Qld, Western Australia and Victoria. The major production region is concentrated in Qld from Caboolture to Eumundi, producing approximately 15,000 tonnes annually, and valued at $140 million (Strawberry R&D update, 2010). In this sub-tropical environment, fruit development is typically from June to October, while in temperate regions i.e., Victoria, fruit development occurs from October to May. With the availability of different cultivars and geographic regions, strawberries are produced year round in Australia.

The majority of cultivars grown in Australia have been bred in California and Florida, USA. These include two of the most popular cultivars grown in southern Queensland in 2010: ‘Strawberry Festival’ (Chandler *et al.*, 2000), here after referred to as ‘Festival’, from Florida and ‘Camarosa’ (Voth *et al*., 1994) from California. Australian bred cultivars account for approximately 15% of those grown within Australia and includes ‘DPI Rubygem’ (Herrington *et al*., 2007) (Strawberry Industry Strategic Plan, 2009).

1.1.3 Strawberry cultivation in Australia

In Australia strawberries are typically grown in open fields in full sun. Several cultivation systems are practised. The most intensive fruit growers use an annual plasticulture system, using the same land continuously for strawberry production for many years (Phillips and Golzar, 2008). This system involves fumigating the soil pre-planting, after which either bare rooted runners or plugs are planted into raised, black plastic covered beds. Typically at the end of the growing season, the plants and plastic are removed and destroyed and a cover crop is established.

For intensive strawberry fruit production, strawberry plants are generally obtained on an annual basis as bare-rooted runners, obtained from runner farms in Victoria, Qld or Tasmania, where the climate allows adequate chill to ensure flowering. Multiplication of plants through tissue culture plays an important role in providing disease and virus free plants to runner farms. These runner farms produce runners that must comply with stringent quality standards (such as 'The Queensland Strawberry Runner Accreditation Scheme' and 'The Victorian Strawberry Certified Runner Scheme') to ensure virus and pest free runners.

1.1.4 Disease management

Strawberry plants are susceptible to a variety of fungi, nematodes and viruses that can cause serious damage and economic losses (Hancock, 1999). Pre-plant fumigation has played a vital role over the past few decades in the control of soil-borne pathogens, nematodes and weeds in strawberry runner beds and for fruit production. Fumigation of runner beds is considered important so that the build-up and subsequent transfer of soil-borne pathogens is minimized. If fungal matter on plants and debris is not effectively eradicated, clean fruit production fields could become infested. In Australia, a zero tolerance is prescribed by the Australian strawberry runner schemes for Fusarium wilt and verticillium wilt, however, other diseases may be present at a low incidence e.g., anthracnose caused by *Colletotrichum gloeosporioides* (Hutton and Gomez, 2010).

1.1.5 Fungal diseases

Important fungal and fungal-like pathogens of strawberry include: *Colletotrichum* spp*., Fusarium* spp*.*, *Macrophomina* spp., *Phytophthora* spp., *Pythium spp.*, *Rhizoctonia fragariae* and *Verticillium* spp. These are considered major diseases worldwide (Nemec, 1975; D'Ercole *et al.*, 1989; Tezuka and Makino, 1991) and cause serious losses to production each year in Australia (Golzar *et al.*, 2007; Mattner *et al.*, 2008; Hutton and Gomez, 2010). Chemical control for strawberry fruit production is used for most fungal diseases (pre and post planting); however for Fusarium wilt presently there is no chemical control available post planting.

Fungal diseases generally occur in wet, warm weather and are responsible for damage to crown, root, fruit, flowers and leaves. Plant-pathogenic fungi can become more prominent, and infection more severe during periods of prolonged wet conditions; anaerobic conditions around the roots can lead to infection by pathogenic fungi (Hancock, 1999). Many of the agronomic practices that are used in the strawberry annual

plasticulture system (e.g., replanting into used plastic, ratooning, excessive irrigation or irrigation with infested water, use of susceptible cultivars and the monoculture system) provide a favourable environment for disease development, putting susceptible genotypes at risk of infection. As a means of cost and labour reduction some growers use ratooning or the practice of replanting into used plastic. Ratooning is the cutting back of plants at the end of the growing season to recycle the plant for the next season. It is estimated that ratooning in Qld accounted for approximately three million plants producing fruit in 2011 (Strawberries Australia Inc, 2012). If soil fumigation is not used, the potential for inoculum build-up and a higher incidence of soil-borne diseases is high, and is exacerbated by a lack of rotation.

Strawberry production has relied heavily on pre-plant fumigation and chemicals as the major strategy for pest and disease control. The development of high yielding varieties dependent on fumigation to maximise yields has dominated the objectives in breeding programs (Mattner, 2005). However, this short term focus and a lack of understanding of the real impact of diseases has resulted in the provision of cultivars dependent on agrochemicals to sustain their yield. With the withdrawal of methyl bromide and more consideration for environmental sustainability, breeding approaches are required to supply a more sustainable strawberry industry in the future.

1.1.6 *Fusarium oxysporum*

Fusarium wilt disease is caused by pathogens of *Fusarium oxysporum* Schlechtendahl emend. Snyder and Hansen (Snyder and Hansen, 1940) and comprise major soil-borne fungal pathogens of many important crops including: vegetables (Walker, 1953), ornamentals (Di Pietro *et al.*, 2003), palms (Priest and Letham, 1996) and banana (*Musa* spp.) (Stover, 1962). *F. oxysporum* and its distribution have been researched and documented as a result of the economic damage caused worldwide to food and fibre crops (Booth, 1977). Specific strains of *F. oxysporum* are pathogenic to specific hosts or a limited number of host species. This relationship has been termed *forma specialis* (f. sp.). There are over 120 *formae speciales* of *F. oxysporum* that have been identified and described, and within some *formae speciales,* subgroups termed ‘races’, pathogenic to a specific host or small number of host plants, are described (Booth, 1975; Correll, 1991). However, it is currently unknown whether physiological races of *Fof* exist to cultivars currently in use in Australia.

1.2 *Fusarium oxysporum fragariae*

Fusarium wilt of strawberry is caused by *Fusarium oxysporum* f. sp. *fragariae*. This disease was first observed and identified in Qld by Williams and Winks in 1962 (Winks and Williams, 1965). Since this first report, Fusarium wilt of strawberry has been confirmed in many countries including: Japan (Takahashi *et al.,* 2003; Mori *et al.,* 2005), Mexico (Davalos-Gonzalez *et al.*, 2006), Korea (Nagarajan *et al.*, 2006), China (Zhao *et al.*, 2009), Spain (Arroyo *et al.*, 2009) and the USA (Koike, 2009).

Recently, in California, outbreaks of *F. oxysporum* and *Macrophomina phaseolina* caused the death of strawberry plants from several areas in the major production regions (Koike, 2009). Plant losses and disease severity from both pathogens were greatest for cultivars Camarosa and Albion (Koike, 2009). In 2007, *Fof* was first reported in a soil-less culture system in Huelva in south-western Spain (Arroyo *et al.,* 2009). In Australia, outbreaks of Fusarium wilt have negatively impacted strawberry fruit production with 10% of plant death in Queensland and 50% in the Perth district of Western Australia (WA) (Golzar *et al*., 2007; Phillips, 2008; Fang *et al.,* 2012) being attributed to *Fof*.

F. oxysporum is a soil inhabitant, capable of surviving indefinitely as a saprophyte. The species *F. oxysporum* is asexual, producing three types of spores; microconidia, macroconidia and chlamydospores (Nelson *et al.*, 1983). Chlamydospores are produced within plant tissue or by soil-borne mycelium, and can remain viable in the soil for many years (Smith and Snyder, 1975; Vakalounakis and Chalkias, 2004). Both micro-conidia and macro-conidia are produced directly by the mycelium and facilitate short-term survival of the fungi; micro-conidia are commonly produced within the vessels of infected plants and macro-conidia on the surface of plants (Burgess, 1981). The pathogen survives in infested soils, plant tissue (as mycelium) and in the air and water (as spores) (Cantrell and Betancourt, 1995). Dispersed by wind, water, soil, runners and debris, infected plants and by cultural operations and practices (Burgess, 1981; Cooke *et al.*, 2009; Snoddy, 2010), the pathogen is difficult to contain or eradicate.

Figure 1.1 Strawberry plants grown in *Fof* infested soil; diseased plants show wilting and collapse of plant.

Figure 1.2 Strawberry plant in field showing *Fof* symptoms

1.2.1 Disease process

Disease progress of *F. oxysporum* is complex, requiring several processes for successful infection and development. Infection is first initiated by recognition of plant root exudates (e.g. flavonoids, phenolics, sugars and amino acids) released into the rhizosphere (Steinkellner *et al.*, 2005), after which germination of conidia and growth of hyphae or germ tubes, predominantly in the root hair zone, penetrate the root epidermis. Hyphae colonise the epidermal cells and the cortex and enter the xylem vessels, spreading to colonise and ultimately block the vascular system, causing the plant to wilt (Lagopodi *et al.*, 2002; Xiao-min *et al.*, 2011). Wilting is believed to be caused not only by the accumulation of mycelium in the xylem causing water stress, but also by toxin production and host defence responses such as tyloses and gels (Nemec, 1995; Rep *et al.,* 2002). Disease severity varies between host species but is typified by wilting, stunting and vascular discolouration.

1.2.2 Symptoms of *Fusarium oxysporum fragariae*

Symptoms of Fusarium wilt of strawberry are typical of other wilt diseases caused by *F. oxysporum*, characterised by stunting of younger leaves, lesions on the petioles, necrosis of the roots, rapid wilting, and the total collapse and death of the plant (Figure. 1.3). The period from infection to the death of a plant can be as quick as two months.

Figure 1.3 Progression of disease response in strawberry plant infected with *Fof*. Photos show healthy plant (far left) declining to plant death (far right).

Fof damage is greatly influenced by climatic factors and disease spread and severity often increases with the onset of warm temperatures. Once infected, and if conditions are favourable (wet and warm weather), plant decline is usually imminent. However, with the onset of cooler, dryer conditions the disease progress can slow or even halt. With these conditions, the plant can produce new growth, the central leaves may remain healthy while the remaining leaves may slightly roll and yellow (Broadley *et al.,* 1988). However,

with the return of hot weather the disease can take hold and spread rapidly (Winks and Williams, 1965).

In strawberries, *Fof* grows within the water-conducting tissues of the crowns and leaves, blocking translocation of water and minerals. When cross sections of the crowns of infected *Fof* plants are examined, the disease is evident by red, brown or black discolouration of the vascular and cortex tissues, roots and xylem vessels, and by crown rots (Figure. 1.4).

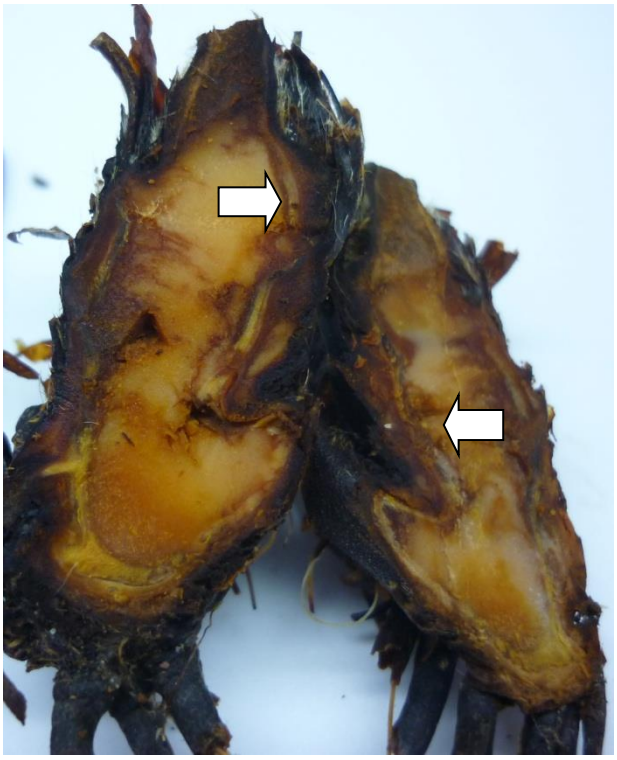


Figure 1.4 Strawberry crown cut longitudinally; exposing vascular tissue discolouration (shown by arrows).

1.2.3 Identification of isolates

Fof can be isolated for identification by samples taken from discoloured sections of the vascular system within the crown of infected plants (Figure. 1.4). When plated on ¼ strength potato dextrose agar media, colonies are usually fast growing with aerial mycelium. The thallus colour ranges from whitish to shades of pink and purple. Microconidia are more prominent and are hyaline, fusiform or ovoid. The macroconidia are hyaline, septate, and sickle or banana shaped (Booth, 1977). To test if isolates obtained from infected strawberry plants are pathogenic strains of *F. oxysporum*, the isolate can be subjected to pathogenicity tests to satisfy Koch's postulates (Falkow,

1988). Identification and diagnosis of *F. oxysporum* strains however is difficult using phenotypic characters and *F. oxysporum* has the potential to mutate in culture and the occurrence of mutants makes identification based on pathogenicity difficult.

Many *formae speciales* are polyphyletic, and an isolate of a *forma specialis* can be more closely related to a member of another *forma specialis* than a member of its own group (O'Donnell *et al.*, 1998; Thangavelu *et al.*, 2012). Identification and knowledge of the variations within the subgroups of the *forma specialis* is vital for breeding for resistant strawberry cultivars. To get an indication of the genetic relationships within or between populations of *F. oxysporum*, vegetative compatibility groups (VCG), volatiles produced, and molecular techniques have been used (Correll *et al.*, 1986; O'Donnell *et al*., 1998; White *et al.*, 1990; Moore *et al*., 1991; Baayen *et al.*, 2001; Martinez-Culebras *et al.*, 2002; Zhou and Everts, 2006).

Vegetative compatibility tests are a comparatively simple and relatively inexpensive means for characterising variation in genetically isolated asexual populations. This technique has been used to study populations of many *Fusarium* species including *F. oxysporum* Schlecht ex. Fr. f. sp. *cubense* (Ploetz and Correll, 1988). Isolates belonging to the same VCG are vegetatively compatible with each other, confirmed by the ability to form a prototrophic heterokaryon, and are often clonally derived from populations of a *forma specialis* (Kistler *et al.*, 1991; Leslie, 1996). PCR techniques have played a major role in the detection of relatedness among strains of *F. oxysporum* and molecular analyses has provided markers able to detect and differentiate pathogenic strains (Gerlach *et al*., 2000; Bogale *et al.*, 2006; Baysal *et al.*, 2010).

Understanding the genetic diversity within *F. oxysporum* populations is important in the breeding and selection process, enabling new progeny to be tested against a wide range of pathotypes. Of major importance to resistance breeding is the knowledge of regional variation in cultivar response to pathogens and also variation in isolate pathogenicity (Hancock, 1999). In a study examining 22 *Fof* isolates from Korea, eight distinct clusters were identified by molecular studies using RAPD and rDNA restriction fragment length polymorphism (RFLP) analysis (Nagarajan *et al*., 2004). In WA, differences in disease severity induced by eight isolates of *F. oxysporum* collected from strawberry plants within WA were recently reported by Fang *et al.* (2011). However to date no studies have been reported examining variation in *Fof* isolates across Australia.

1.2.4 Significance of Fusarium wilt Australia

Soil-borne diseases including Fusarium wilt have until recently been kept in control by pre-plant fumigation using methyl bromide/chloropicrin mixtures, however, Fusarium wilt has, in recent years, become more prominent. *Fof* has been reported as one among the most virulent pathogens causing crown and root diseases of strawberry in WA (Fang *et al.*, 2011), with death of up to 50% of plants in the Perth district being attributed to *Fof* (Golzar *et al.*, 2007; Phillips, 2008; Fang *et al.*, 2010). In the production district of WA, crown and root diseases of strawberry are considered a serious problem to strawberry production (Golzar *et al.*, 2007). In the 2005 season, growers in WA reported unusually high levels of plant death in strawberry crops in both fumigated and non-fumigated soils. As a result, a survey (Department of Agriculture and Food, 2005-2006) was undertaken to identify the causes and severity of plant deaths. *Fof* was identified as the predominant pathogen responsible for much of the plant death. In some fields the cultivars 'Camarosa' and 'Gaviota' suffered up to 60% mortality (Golzar *et al.*, 2007). Over a two-year period *Fof* was isolated from 70% of samples tested (Phillips, 2008). In 2008, a further survey showed that plant decline/death occurred in both non-fumigated and fumigated field beds. Where fumigation had been applied correctly, *Fof* and other soil-borne pathogen populations increased from August to October (Fang *et al.*, 2010). This was believed to be favoured by the onset of warmer weather. *F. oxysporum* was most frequently isolated from crowns and was the dominant pathogen associated with crown discoloration of strawberry. Up to 41% of total isolates tested were *F. oxysporum,* isolated either as the sole species or one of several isolated from infected crowns and roots (Fang *et al.*, 2010).

1.3 Disease management challenges and strategies

With the increasing threat from crown and root diseases, strawberry growers and breeders require knowledge not only on pathogen occurrences, changes in pathogen virulence, damage levels and the economic impact, but also the effectiveness of control methods. A major problem facing strawberry growers using the plasticulture system is soil-borne disease control. Since the 1970s growers have relied on methyl bromide as the major fumigate for disease control, and until the phase-out of methyl bromide in Australia in 2005, under the 'Montreal Protocol on Substances that Deplete the Ozone Layer', many soil-borne fungal diseases of strawberry including Fusarium wilt were successfully controlled (Hutton *et al.*, 2001). Methyl bromide has been a fundamental

component in the production system for both strawberry runner and fruit for the past 40 to 50 years due to being robust and giving increased growth response in the absence of any detectable pathogens and weeds (Munnecke, 1967; Matthiessen and Kirkegaard, 2006; Porter *et al.*, 2006). It is estimated that the use of methyl bromide prevented yield losses in strawberry production of up to 35% from diseases and weeds (Mattner, 2005). Due to these merits, pesticide free management strategies were given little attention.

Since the phase-out of methyl bromide, soil-borne disease management has become a major concern for strawberry production. As such, research has concentrated on alternative soil disinfestation strategies (Mattner, 2005; Shennan *et al.*, 2009; Hutton and Gomez, 2010). Although Telone C35/chloropicrin (TC35) has been adopted in Australia as the pre-plant fumigant substitute to methyl bromide, in field trials TC35 was less effective than methyl bromide, with 59% and 84% respectively of plants remaining healthy (Menzel *et al.,* 2006). Hutton and Gomez (2010) tested other fumigant replacements for their efficacy to control soil-borne pathogens. All fumigant replacements tested were less effective than methyl bromide and each fumigant tested controlled but did not eradicate Fusarium wilt. Fumigation options were found to be either more expensive or give an inadequate extent of control, i.e., they are effective for some diseases but are less effective on others (Hutton and Gomez, 2010). A more sustainable approach to disease control, involving the use of resistant cultivars, is urgently needed (Cooke *et al.*, 2009).

1.4 Plant-pathogen interactions

Plant disease results from an interaction between a host species, pathogenic organism, and environmental conditions. Plants defend themselves to pathogen entry and respond to pathogen attack in a variety of ingenious and complex ways. These include structural or chemical barriers, rigid cell walls, receptors that detect pathogens and activate inducible defence responses, the production of toxic compounds and the detoxification of pathogen toxins. These ultimately will influence host resistance, tolerance, or susceptibility to pathogen attack.

In order to elicit defence mechanisms that impede infection, plants use an array of signalling mechanisms in defence response that influence the disease outcome. Plant innate immunity can be prompted by microbe associated molecular patterns (MAMP), recognised by the plants to induce MAMP-triggered immunity (MTI), and effector recognition by plant resistance *R* gene products that may result in effector-triggered

immunity (ETI). Defence responses may signal pathways that lead to a hypersensitive response (HR) mediated by salicylic acid-, ethylene- and/or jasmonic acid-dependant signalling pathways (Muthamilarasan and Pradad, 2013; van Loon *et al.,* 2006; Montesano *et al.,* 2003).

The mechanism of subsequent resistance/susceptibility is based on a gene-for-gene model (Flor, 1956), in which specific resistance gene(s) (*R*) products in the host recognise specific pathogen avirulence (*Avr*) gene products in the pathogen. If gene-for-gene interactions are established, pathogen presence is perceived by plants through recognition of molecules produced or released by the pathogen known as elicitors. Pathogen elicitors secrete a variety of *Avr* gene products which are recognised by specific host receptors located on plasma and sub-cellular membranes. Elicitors are perceived as biological signals that stimulate and activate plant defence (Montesano *et al.,* 2003). Elicitors consist of two types; general and race-specific. General elicitors induce defence responses in both host and non-host plants, while race-specific elicitors trigger defence only in specific host cultivars.

Following recognition between host and pathogen by plant resistance (*R*) factors and pathogen virulence factors (avirulence genes), both host and pathogen release an assortment of biochemical substances, structures, and pathways. A ‘compatible’ response, or plant failure to recognise pathogen virulence factors, will not induce defence responses. This may result in infection and the progression of disease. An ‘incompatible’ interaction initiated by a plant carrying an *R* gene and pathogen carrying the complementary *Avr* gene activates a signal transduction pathway to induce plant defence responses. Following the induction of host defence responses by a compatible interaction PR-proteins are elevated, enhancing resistance to other pathogens, this response is known as systemic acquired resistance (SAR). When SAR is activated a HR may occur.

Many pathogens possess host-specific toxin (HST) genes and or avirulence genes. In the situation of necrotrophs or hemi-biotrophs, pathogens with the HST genes can secrete protein effectors which interact with the host carrying a corresponding toxin-sensitivity gene to initiate disease; in the same manner as an *Avr* gene in the gene-for–gene model. A compatible HST will result in disease while and incompatible HST with result in resistance (Stukenbrock and McDonald, 2009).

Fusarium species use both general (e.g., cellular signalling pathways, fungal enzymes) and pathogen-specific (HST, secreted effectors) pathogenicity mechanisms to invade a

host (Ma *et al.,* 2013). The production of toxins and other secondary metabolites (e.g. trichothecenes) contribute to plant pathogenicity. In *F. oxysporum,* specialised pathogenicity genes described as *SIX*-genes (secreted in the xylem) have been shown to be involved in a gene-for-gene interaction with host species (Rep *et al.,* 2004; Ma *et al.*, 2010). These virulence genes can be host-specific, e.g., *SIX4* is present in race 1 of *F. oxysporum* f. sp. *lycopersici (Fol)*, but absent in races 2 and 3 (Lievens *et al.,* 2009).

Expression of susceptibility (or resistance) in a host to a pathogen is strongly affected by the interaction between environment, genotype and pathogen. The outcome of disease development and plant decline will ultimately depend on the speed, magnitude and effectiveness of plant defensive mechanisms (van Loon *et al.*, 2006), and the inherent resistance of the genotype.

1.5 Breeding for disease resistance in strawberries

Host plant resistance through breeding is an effective and economical way to control plant disease in crops (Porter *et al.*, 1997; Rudd *et al.*, 2001). The use of resistant cultivars is environmentally benign, compatible with organic/low pesticide systems, lessens the need for chemicals and subsequent residue levels, and is overall a more sustainable approach to disease control.

The genetics and cytology of the modern strawberry is complex (Hancock, 1999), however varying degrees of susceptibility of hosts to pathogens and an abundance of resistance genes in plants (Hermann *et al.*, 2006; Korbin, 2010) enables breeders to transfer resistance along with other sought after traits into new cultivars. The cultivated strawberry *Fragaria* × *ananassa* is considered a complex polyploid, derived from as many as four different diploid ancestors. *Fragaria* × *ananassa* is an alloploid*;* but has been shown to act a diploid by cytological observations *(*Senanayake and Bringhurst, 1967*;* Davis and Yu, 1997*;* Haymes *et al.,* 1997*;* Sargent *et al.,* 2004*;* van de Weg *et al.,* 2006*)* and in segregation studies with co-dominant molecular markers, isozymes and closely linked AFLP and RAPD markers (Sargent *et al.,* 2004, Degani *et al.,* 1998; Haymes *et al.,* 1997). The amphidiploid nature of *Fragaria* × *ananassa* allows the use of standard mapping and gene quantitative trait loci (QTL) and bulked segregate analysis (Darrow, 1966; Bringhurst, 1990; van de Weg *et al.,* 2006; Sargent *et al.*, 2009).

The genetic base in the cultivated strawberry is relatively narrow (Sjulin and Dale, 1987), however high levels of heterozygosity in the strawberry genome and the hybrid nature of

Fragaria × *ananassa* (Hancock, 1999; Maas, 2004; Naqvi, 2004) makes breeding for disease resistance a viable option to disease management for strawberry. Resistance breeding has been successful in strawberry for: red stele root rot, Phytophthora crown rot, Verticillium wilt, powdery mildew, Alternaria black leaf spot, anthracnose (black spot) and Fusarium wilt (Hancock *et al.*, 1990; Nelson *et al.*, 1996; Naqvi, 2004). There is however limited information available on the genetics of resistance to *Fof* (Maas and Galleta, 1997). From Mori *et al.,* (2005) it is understood that both qualitative and quantitative genes are involved in *Fof* resistance and polygenic heredity is assumed by Davalos-Gonzalez *et al.*, (2006).

1.6 Sources of resistance

Strawberries can be grown in any arable region of the world due to the availability of cultivars derived from a variable gene pool and also to cultural practices (Hancock, 1995). Genetic diversity is essential for plant breeding, providing germplasm to develop higher-yielding, disease resistant cultivars with improved crop productivity. Wild relatives, heritage and current cultivars are resources available for breeding programs.

There is currently a lack of *Fof* resistant cultivars or knowledge of cultivar resistance. To date, only three cultivars of strawberry have been bred with the aim of *Fof* resistance. These were bred in Japan by traditional screening methods for use in open culture farming in the cooler regions of northern Japan (Takahashi *et al.*, 2003). Several cultivars and wild species of strawberry have been identified as having resistance or tolerance to *Fof*. For example, the cultivar ‘DPI Rubygem’ has been identified as having resistance to *Fof* (Herrington *et al.*, 2007) and wild clones of *Fragaria chiloensis* growing in California were selected for their resistance to *Fof* (Davalos-Gonzalez *et al.*, 2006). The cultivar Festival is one of the major cultivars grown in Australia, and from trials conducted at Maroochy Research Station in Nambour, Qld, is considered resistant to *Fof* (Hutton *et al.*, 2006).

The identification and development of host plant resistance to important pathogens has become an important goal of many crop breeding programmes (Maas and Galletta, 1997; Hermann *et al.*, 2006). This has intensified since the phase-out of methyl bromide and will be an ongoing process, requiring breeders to screen germplasm from both cultivars and wild strawberry species for the identification and transfer of resistance genes.

1.7 Breeding strategy for resistance

Breeding for resistance in crops can be complex. The incorporation of host plant resistance to diseases of strawberries involves manipulating or changing the genome of the plant to attain pathogen resistance, while maintaining fruiting and other desirable traits. Commonly, varietal improvement programmes have enhanced genetic resistance in strawberry by identifying resistant germplasm for backcrossing into elite parents. Desirable traits to be incorporated into commercial cultivars have often been highly heritable (Hancock, 2008) even though under polygenic control, making recurrent selection strategies viable.

1.8 Traditional plant breeding strategy

The traditional breeding method typically used for host plant resistance to a pathogen involves pathogenicity testing of new selections/cultivars to evaluate host/pathogen responses and involves crossing selected parents with suspected resistance and desirable traits and then several backcrosses of progeny to the agronomical desired parent. Glasshouse and field screening and selection among cultivar and wild species germplasm are conducted to identify desirable traits (alleles) that can then be used as parents. Recurrent selection is a cyclical selection plant breeding method that is used for the improvement of quantitatively inherited traits and identification of superior lines. This method increases the frequency of desirable traits (alleles) using one or a combination of crosses (e.g. full-sib, half sib, or inbred) from a population. From this process the study of progeny for phenotypic distributions of populations, the number of relevant genetic loci, degree of dominance, additively, heterosis, and gene/environment interactions can be undertaken (Young, 1996).

1.9 Evaluation of disease resistance

The identification and incorporation of host plant resistance relies on a proficient and reliable germplasm screening process to determine germplasm susceptibility under disease exposure and pressures. This requires a good understanding of variability in pathogen populations and virulence factors (Russell, 1978; Lebeda and Švábová, 2010). Pathogen variation is important to the effectiveness of screening, as pathogen genotypes will respond as compatible or incompatible to specific hosts. The identification of virulence variation by pathogenicity testing on several genotypes of defined resistance or

disease phenotype is necessary to provide suitable inoculum for screening (Russell, 1978).

Various methods have been used for screening of strawberry for resistance to fungal diseases. Methods used for evaluating strawberry genotypes for resistance to *Fof* have included the evaluation of plant response to *Fof* under open field infested soils (Toyoda *et al.*, 1991; Takahashi *et al.*, 2003), inoculation of plants under glasshouse conditions; either at early seedling stage (Takahashi *et al.*, 2003) or at mature plant stage (Koike, 2009), and evaluation of resistance in solution culture (Kuroda and Tomikawa, 2001). In Australia, varietal field screening for susceptibility to *Fof* has successfully been achieved by Hutton *et al*. 2004 (unpublished data). Glasshouse inoculations of strawberry with *Fof*, using a spore concentration, have been practiced by Winks and Williams (1965) and Fang *et al.* (2011). All methods resulted in adequate disease development and the decline and death of susceptible genotypes.

1.10 Heritability

Studies of inheritance have been undertaken to analyse how much of the phenotypic variation in a population is influenced by genetic factors to provide insights into the suitability of the resistance trait for breeding (Korbin, 2010). Genetic improvement of specific traits in plants can be obtained by knowledge of the parent's performance, progeny testing, combining ability estimations, observed selection responses, or by breeding values. There are several methods and tools used to provide information to plant breeders about heritability, these include specialised mating designs (e.g., full, partial or incomplete diallel design) and statistical models used to identify the type of gene action for specific resistance loci (e.g., example dominant, recessive, additive) (Young, 1996). Predicted genetic models for heritability can be used to assess screening results and statistical models can generate individual breeding values and so determine suitable parents from an observed sample of progeny.

Strawberry breeders have used a number of systems to predict heritability. Offspring-parent regression heritability has been used by Shaw (1989), who estimated heritability for yield and appearance in strawberries using data collected for strawberry seedling from biparental progenies subjected to three cold storage treatments. Significant differences were found among heritability estimates, detected from scale differences and ranked changes over test environments. Results from this method led to both over and under estimations of predicted gain. Estimations based on variance components have

been found more reliable. Gupton and Smith (1991) tested strawberry progeny against *Colletotrichum* spp. to estimate the genetic variances (both narrow and broad sense) by variance components. The narrow-sense heritability estimates were considered sufficient enough to produce gains from recurrent selection. This was confirmed with increased levels of resistance with successive cycles of selection (Gupton and Smith, 1991).

Knowledge of allele effects and frequency is of great importance to predict progeny value. The additive genetic effects or breeding value of an individual are passed on from parent to offspring, therefore the additive genetic variance accumulates within families. From screening and analysis of a population, two types of heritability can be estimated; broad-sense heritability, which is the ratio between total phenotypic and genetic variances, and narrow-sense heritability, which is the ratio between the additive genetic and total phenotypic variances. The latter is more useful in plant breeding due to genetic outcomes of progeny being dependent on the additive genetic variance.

Breeding values have been used in many breeding programs to increase the frequency for the desired phenotypic outcomes in progeny (Kennedy, 1981; Shaw and Sacks, 1995; de Souza and Byrne, 2000; Hardner *et al.*, 2012). The breeding values of each individual from a breeding population are obtained from 'best linear unbiased predictions' (BLUPs) (Henderson, 1984). BLUPs are an analysis of random effects generated from linear mixed models, and are useful for identifying best performing lines, the suitability of lines as parents, and their general combining ability (Falconer and Mackay, 1997; Oakey *et al.*, 2006). For strawberry, Davik and Honne (2005) used a mixed model incorporating pedigree information to estimate variance components and heritabilities for resistance to powdery mildew. They found narrow-sense heritability increased with the addition of pedigree information. Incorporating pedigree data into the model increases the predictive ability of phenotypic outcomes. Information on the relatedness of the genotypes can give better estimates of total genetic effects and predicted breeding values, therefore enabling better predictions about progeny response to selection (Davik and Honne, 2005; Crossa *et al.*, 2010).

Despite extensive knowledge on Fusarium wilt diseases in other crops, little is known of the genetics of resistance in strawberry. Information is required about the heritable variation and heritability of *Fof* resistance in strawberry so that favourable genotypes can be identified and utilised in breeding programs. Strawberry is an ideal crop for phenotypic selection using predicted breeding values as the short generation interval allows for

progeny measurements to be performed and available for analysis within one season. Knowledge of the genetics of resistance in strawberry to *Fof* can assist breeders to make more effective breeding decisions.

1.11 Aim of this study

The potential for crop losses due to Fusarium wilt in the major strawberry producing areas of Australia has only recently become evident. Using resistant cultivars is a sustainable approach to the management of Fusarium wilt disease.

To enable strawberry breeders to develop cultivars with included *Fof* resistance, it is important to determine the variability in the virulence and genetics of Australian *F. oxysporum* isolates pathogenic to strawberry. Furthermore, it is important to identify strawberry genotypes with high levels of resistance to *Fof* and understand how to transmit the resistance. Therefore the overall objectives of this study are to investigate the diversity of *Fof* in Australia, to identify the resistant plant genotypes, and to determine the inheritance of plant resistance. The aim of this study is to compare the pathogenicity of a range of *Fof* isolates, investigate inoculum application methods for adequate disease development, identify sources of plant resistance, and identify plants suitable as parents in further breeding trials. This research was conducted as an aid for the development of new elite subtropical strawberry cultivars incorporating *Fof* resistance.

2.0 Chapter 2 Genetic variation among Australian isolates of *Fusarium oxysporum* from strawberry

2.1 Introduction

The recent occurrence of high levels of plant death from Fusarium wilt in WA (Phillips and Golzar, 2008) and southeast Queensland (Hutton and Gomez, 2010), following the phase-out of methyl bromide, demonstrates the potential for Fusarium wilt to become a major threat to the strawberry industry in Australia. An alternative method of disease management is needed to combat the high incidence of Fusarium wilt. Breeding strawberry cultivars with improved resistance would greatly enhance the success and sustainability of strawberry production in Australia. For the breeding and selection of resistant cultivars, knowledge of the diversity of the pathogen is critical to ensure that new cultivars are tested against as wide a range of potential pathotypes as possible. Information on the genetic variation among *Fof* populations across Australia as a whole is unknown. Fang *et al.* (2011) recently reported significant differences in disease severity induced by eight isolates of *F. oxysporum* collected from strawberry plants within WA. An assessment of the virulence of a broader range of Australian isolates of *Fof* would allow the identification of highly virulent or diverse strains, which in turn can be used as inoculum in selection of resistant strawberry genotypes in breeding programmes.

F. oxysporum is sub-divided into *formae speciales* (form species) based on host specificity of isolates. The *forma specialis* of a *F. oxysporum* isolate has been typically assigned by pathogenicity tests and the ability of the isolate to cause disease symptoms. The pathogenicity of the pathogen is determined either by plant survival/death or by virulence [the degree of pathogenicity of a given isolate (Agrios, 2005)], measured on a disease severity scale/index. Strains within *formae speciales* that are selectively pathogenic to certain cultivars of one or more plant species can be further classified into physiological 'races' (Armstrong and Armstrong, 1981; O'Donnell *et al*., 1998; Baayen *et al.,* 2000). It is currently unknown if within the *Fof* population whether physiological races exist to cultivars currently in use in Australia or elsewhere.

Conventionally, the genetic diversity of *F. oxysporum* has been assessed by analysis of vegetative compatibility groups (VCGs), using nitrate non-utilising (*nit*) mutants and pairing isolates with each other to verify VCG (Cove, 1976; Puhalla, 1985; Correll *et al.*, 1987). Nagarajan *et al*. (2006) identified three major VCGs and one incompatible group among 22 isolates of *Fof* collected from different strawberry cultivating areas in Korea. Hyun *et al*. (1996a), also from Korea, assigned 32 isolates of *Fof* using VCG testing, into

four major VCGs. In Australia, no studies on VCGs of the *Fof* population have been reported.

Molecular methods have recently enabled a more detailed analysis of the genetic diversity and associations within and among *formae speciales* (Sarfatti *et al.*, 1991; Baudracco-Arnas and Pitrat, 1996; Gerlach *et al.*, 2000; Swetha Priya and Subramanian, 2008) which has enabled a closer correlation with host range compared to that provided by phenotypic or morphological methods (Taylor *et al.*, 2000; O'Donnell *et al.*, 2004). However as yet there are no reports on the molecular analysis of populations of *Fof* from Australia.

Molecular techniques have been used in studies from Korea to resolve variation among *Fof* isolates and for phylogenetic analysis. In a phylogenetic study, Hyun and Park (1996b) used random amplified polymorphic DNA (RAPD) PCR to separate 24 isolates of *Fof* into two distinct clades but were unable to distinguish among *formae speciales*. Nagarajan *et al.* (2004) used RAPD and restriction fragment length polymorphism (RFLPs) of intergenic spacer (IGS) region of rDNA to study variation among isolates of *Fof* from various areas in Korea. They concluded that a high level of genetic variability existed in *Fof,* identifying eight distinct clusters, and noted dissimilarities between isolates from different geographical locations in Korea. In another study, Nagarajan *et al*. (2006) tested isolates of *Fof* by VCG, RAPD, and pathogenicity testing, and noted a relatively high correlation existed among VCG and RAPD, and virulence.

The mitochondrial DNA has been reported to have a higher rate of evolution than nuclear DNA (Brown *et al.* 1979), and therefore useful for high-resolution of relationships among and within lineages. A number of studies have shown the mitochondrial rRNA small subunit (mtSSU) and also translation elongation factor 1-α (EF-1α) gene region to be useful for revealing genetic and evolutionary relationships among and within *formae speciales* of *F. oxysporum.* Rahjoo *et al.* (2008) were able to identify unknown isolates of *F. oxysporum* f. sp. *verticillioides (Fov)* by using the EF-1α gene region. O'Donnell *et al.* (1998) demonstrated that combined EF-1α and mtSSU gene regions were excellent for resolving relationships within the *F. oxysporum* complex. In a phylogenetic study, Fourie *et al.* (2009) determined the genetic relatedness among and within VCGs of *F. oxysporum* f, sp. *cubense (Foc)* and other *formae speciales* and non-pathogens using combined EF-1α and mtSSU datasets. In another phylogenetic study, Bogale *et al.* (2006) were able to group 18 *formae speciales* of *F. oxysporum* into three distinct

lineages using EF-1α and mtSSU sequence data. These studies reveal that the EF-1α and mtSSU gene regions are suitable to separate isolates of *F. oxysporum* into statistically supported groups.

The objectives of this study were to evaluate pathogenicity and genetic variations among *F. oxysporum* isolates collected from regions within Australia, by means of pathogenicity testing, VCGs, and partial sequencing of the EF-1α and the mtSSU gene regions. The results will be valuable in understanding the genetic diversity of Australian isolates and useful for the breeding of strawberry cultivars resistant to Fusarium wilt.

2.2 Materials and methods

2.2.1 Fungal isolates

All of the isolates used in this study (Table 2.1) were obtained from the crowns of symptomatic strawberry plants collected from the 1960s through to 2009 within Australia. The majority of isolates came from regions within southeast Queensland where strawberry production is most intensive and from WA where heavy infestations have been reported (Golzar *et al.,* 2007) (Table 2.1). All isolates had been identified as *F. oxysporum* based on spore and colony morphology. The *forma specialis* of several isolates were unknown (untested). Single-spore isolates were stored on filter paper at the Maroochy Research Facility, at Nambour. *F. oxysporum* f. sp. *zingiberi* (BRIP39298) which infects ginger, was included in molecular studies as a comparison/outgroup. Isolates were plated onto 1/4 strength potato dextrose agar (PDA) and incubated at 27ºC for approximately 1 week prior to VCG and DNA analysis, and 3 weeks for inoculum preparation.

Table 2.1 Isolates of *F. oxysporum* used in comparing pathogenicity, VCG, and EF *1α* and mtSSU sequences.

Accession number [x]	Year collected	Geographic origin	Host cultivar
N9054	1962	Victoria Pt. Qld	unknown
N9055	1962	Victoria Pt. Qld	unknown
SA126	1981	Adelaide Hills, SA	unknown
N9103	1989	Nambour, Qld	Tioga
N9551	1990	Palmwoods, Qld	Earlisweet
N10010	1991	Caboolture, Qld	Parker
N10226	1992	Hastey Park, NSW	Torrey
N13581 [z]	2002	Palmview, Qld	Kabarla
N15309 [z]	2005	Wamuran, Qld	Camarosa
N15457 [z]	2005	Nambour, Qld	Pajaro
N15915	2006	Wamuran, Qld	Rubygem
N16004 [z]	2006	Wamuran, Qld	Selva
N16239	2006	Wannerroo, WA	unknown
N16240	2006	Wannerroo, WA	unknown
N16818	2007	Nambour, Qld	unknown
N16893	2007	Beenleigh, Qld	Ventana
N16999	2007	Redlands, Qld	Camerillo
N17203	2008	Stanthorpe, Qld	Treasure
N17337 [z]	2008	South Perth,WA[y]	unknown
N17350	2008	Chevallum, Qld	Rubygem
N18419 [z]	2009	Stanthorpe, Qld	Malibu
N18421 [z]	2009	Stanthorpe, Qld	Cal Gaint 3
N18437	2009	Stanthorpe, Qld	unknown
N18462 [z]	2009	Wanneroo, WA	unknown
N18582 [z]	2009	Nambour, Qld	unknown
N18842	2009	Stockleigh, Qld	Camarosa
N18936	2009	Atherton, Qld	Albion
BRIP39298	1998	Beerwah, Qld	Canton (ginger)

[z]Isolates used in virulence testing
[y]Exact location is unknown
[x]All isolates were obtained from Department of Agriculture, Fisheries, and Forestry, Qld Government, Australia

2.2.2 Plant material

Strawberry plants of the cultivar Kabarla, reported to be susceptible in the south eastern regions of Qld (Hutton *and* Gomez, 2006), were obtained as bare rooted runners from a certified runner nursery. They were planted in 100mm x 140mm pots containing steam-sterilised potting mix composed of double washed river sand and coir (by volume:1:1) with a pre-mixed fertiliser of (g/L) 5.1 nitrogen, 7.2 phosphorus, 4.6 potassium, 60.4 calcium, 0.09 copper, 0.06 iron, 0.32 magnesium, and 0.15 zinc. The potted plants were stored in a shade house, watered, and fertilised as required until established.

2.2.3 Pathogenicity tests

A sample of nine isolates (N13581 N15309, N15457, N16004, N17337, N18419, N18421, N18462, and N18582), collected from southeast Queensland and WA and representing locations of high intensity strawberry production were used for root dip inoculations on the cultivar Kabarla. To validate the inoculation procedure, the isolate N18462 was used as a positive control; it had been isolated from affected plants in an episode associated with a high incidence of crown rot in the 2005 and 2006 seasons in WA (Phillips and Golzar, 2008).

Single-spored isolates of *F. oxysporum* were plated onto 1/4 strength PDA, and incubated at 27ºC for 3 weeks. The spores were collected from culture plates following addition of sterile deionised water by rubbing the agar surface with a glass spreader. The colony morphology was similar for all isolates with the exception of the isolate N16004 that did not have the characteristic of *Fof* colonies, exhibiting thick dark orange mycelium. The spore suspension was then filtered through four layers of cheesecloth. The conidial concentration was determined using a haemocytometer and adjusted to 1×10^6 conidia/mL. Inoculum was used immediately (within 3 to 5 hours) in root-dip inoculations.

Plants (≈ 6 months old) were carefully removed from their pots, the crown and roots washed to remove potting mix and dried with an absorbent cloth. The plants were inoculated in a randomised order with five replicates, by immersing the crown and roots in the inoculum for 10 minutes. Five untreated control plants were similarly immersed in sterile water only. The plants were then firmly placed in their pots using potting medium (previously described in section 2.2.2). Approximately 10mm of sterile gravel (3 to 5mm in diameter) was added around the plant on top of the soil mix to prevent splash. Each pot was then randomly allocated, spaced at ≈25 to 30cm apart, onto a heated bench at 28°C in a glasshouse with natural daylight. Plants were watered to free draining with tap

water daily for up to 5 days and approximately three times per week thereafter, and fertilised at 2 week intervals with 'Yates Aquasol Soluble' fertiliser, at the recommended rates.

2.2.3.1 Disease severity assessment

Disease development was monitored weekly on the individual plants and visual severity ratings taken 8 weeks post-inoculation. Severity of foliar symptoms was assessed on a 0 to 10 disease visual index modified from (Hutton *et al*., 2006) where:

0 = plant healthy, with erect growth and full vigour

1 = plant generally healthy, with smaller canopy and moderate vigour

3 = plant with a slight wilt, with lower leaves affected

5 = plant with a moderate wilt, with the mature leaves collapse but young leaves still healthy

7 = plant with a severe wilt, with most of the plant collapsed and desiccated

9 = plant with a very severe wilt, with the entire plant collapsed and desiccated

10= plant dead

A mean disease severity score for each isolate treatment was calculated across replicates. The degree of virulence (x) to *Fof* on the cultivar Kabarla was determined from the mean disease severity rating by the following scale:

$x \leq 2$ = non-virulent

$2 < x \leq 4$ = slightly virulent

$4 < x \leq 7$ = moderately virulent

$x > 7$ = highly virulent

2.2.3.2 Crown sampling for Fof recovery

Crowns were sampled for *Fof* recovery on two to five plants of each pathogenicity test at 8 weeks post-inoculation. The crowns were washed clean and surface sterilised in 0.5% sodium hypochlorite for 5 minutes and rinsed three times in sterile water. Crowns were cut in cross sections and discoloured pieces (or if healthy, section of vascular tissue) of the crown plated onto 1/4 strength PDA and incubated at 27°C. After 1 week, plates were inspected and analysed using a compound microscope (magnification 400X) for the presence of *F. oxysporum.*

2.2.3.3 Confirmation of pathogenicity tests

To both confirm that the re-isolations obtained from infected strawberry plants were virulent strains of *F. oxysporum* and satisfy Koch's postulates (Falkow, 1988), the re-isolates underwent further pathogenicity tests on six replicate 'Kabarla' plants. Six non-inoculated 'Kabarla' plants were treated as controls. Additionally six plants were treated with the original isolate N17337 as a comparison. The same inoculation procedure and visual assessment was used as that previously described in section 2.2.3.

2.2.3.4 Statistical analyses for pathogenicity

All statistical analyses for pathogenicity tests were performed using Genstat (version 11.1) (VSN International Ltd.). Analysis of pathogenicity was performed using severity rating means taken at 8 weeks post-inoculation, and compared by Analysis of Variance (ANOVA) and on the estimate of Fisher's protected least significant difference test ($P<0.05$).

2.2.4 Vegetative compatibility grouping of *Fusarium oxysporum* isolates

All isolates listed in Table 2.1 with the exception of N18419, N18421 and BRIP39298 were used in vegetative compatibility tests. The technique used to group isolate strains of *F. oxysporum* by vegetative compatibility was as described by Puhalla (1985) and Correll *et al.* (1987). *F. oxysporum* Isolates were plated onto 1/4 strength PDA, and incubated at 27ºC for 1 week. Three biochemically different nitrate non-utilising mutants (*nit* mutants) were obtained from minimal medium (MM) amended with potassium (Puhalla, 1985). Sectors growing from the restricted colonies, recognised by aerial mycelium (Figure 2.3a), were identified by phenotype (Figure 2.3b) produced when a small mycelia plug taken from the growing edge of the sector was transferred onto MM with one of three forms of an nitrogen source, $NaNO_2$, $NaNO_3$ and Hypoxanthine, in the medium. These *nit* mutants were termed *nit* 1, *nit* 3 and Nit M respectively. All combinations of isolate mutants were paired on MM in Petri dishes. Two isolates were paired by placing a mycelia disc of one *nit* mutant strain in the centre with four *nit* mutants of another strain arranged around the edges (Figure 2.3c). Where possible a Nit M was paired with up to four *nit* 1 mutants from each isolate paired. Where a Nit M was not generated, a *nit* 3 was substituted. If able to form a prototrophic heterokaryon with the same strain but different phenotypic class, isolates were considered self-compatible. Combinations of *nit* mutants from all isolates were paired to verify VCGs, determined by the isolate's ability to form a

heterokaryon (Figure 2.3c). If an isolate formed no heterokaryon with any other isolate it was termed a ‘single member’.

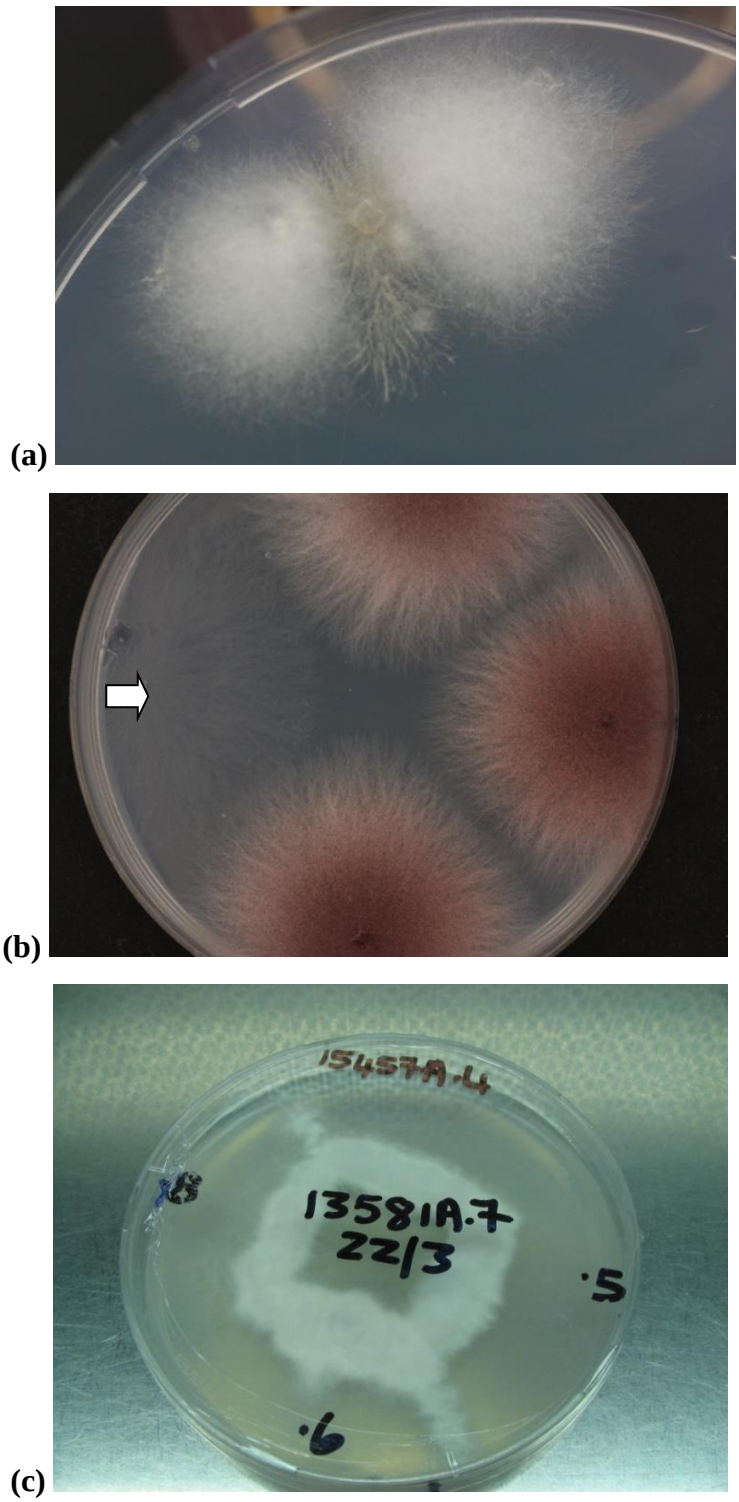


Figure 2.1 (a) Sectors of *F. oxysporum* mycelia generated on MM media amended with potassium chlorate. Aerial mycelium produced from dense tightly packed sectors indicates a *nit* mutant. (b) *Nit* M (left) identified by sparse growth. (c) Heterokaryon formation formed by *nit* mutants belonging to the same VCG.

2.2.5 PCR and partial sequencing of EF-1α and mtSSU

For DNA extraction, isolates listed in Table 2.1 were plated onto 1/4 strength PDA and incubated at 27°C for 1 week. Approximately 50mg of mycelia from each isolate was extricated and collected from culture plates using a sterile scalpel blade. DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions or the Biosprint 15 DNA Plant Kit (Qiagen) on a Biosprint 15 workstation (Qiagen) for EF-1α and mtSSU analysis respectively.

The EF-1α and the mtSSU gene regions were amplified using forward and reverse primers EF-1 and EF-2 as described by O'Donnell *et al.* (1998) and MS1a and MS2a modified primers based on White *et al.* (1990) (Table 2.2). Polymerase chain reactions (PCR) for EF-1α amplification was performed using a thermal cycler (Eppendorf Mastercycler ep), in 25.0 *µ*L reaction volumes containing 12.5 *µ*L MyTaq Red Mix (Bioline, Australia), 0.25 *µ*L 50 µM of each primer (Sigma Aldrich, Australia), and 1.0 *µ*L DNA template (1/5 dilution). Thermocycling conditions were 94°C for 3 min, 35 cycles of 94°C for 45 s, 55°C for 15 s and 72°C 15 s; and a final extension at 72°C for 5 min. A negative control (no DNA template) was included. PCR products were visualised on 1.5 % agarose gel stained with ethidium bromide to confirm expected product size (600-700bp). PCR for mtSSU amplification was performed using an 'Eppendorf Mastercycler ep' in 25.0 *µ*L reaction volumes containing 12.5 *µ*L GoTaq Green Master Mix (Promega), 1.0 *µ*L 10 *µ*M of each primer, and 1.0 *µ*L DNA. Thermocycling conditions were 40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 90 s, and 72°C 10 mins (O'Donnell *et al.*, 2004). A negative control (no DNA template) was included. PCR products were purified by centrifugation using The Wizard® SV Gel and PCR Clean-Up System protocol (Promega) according to manufacturer's instructions.

All sequencing was performed in both directions (both strands were sequenced) to ensure sequence accuracy. The EF-1α PCR products were sequenced using primers EF-3 (internal forward) and EF-22T (internal reverse) (Table 2.2). PCR product purification and sequencing were performed by Macrogen Inc. (Seoul, Korea). Sequence quality was confirmed by using both EF-1 and EF -2, and by visual inspection of the chromatogram using Finch TV (Geospiza). The mtSSU PCR products were sequenced using MS1a and MS2a primers (Table 2.2). Sequencing was performed by the Australian Genome Research Facility (Brisbane, Australia).

Table 2. 2 PCR and sequencing primers used to generate PCR products and for DNA sequencing.

Gene primer	Locus	Primer sequence (5′ to 3′)	Reference
EF-1	EF-1α	ATGGGTAAGGARGACAAGAC	O'Donnell *et al*., 1998
EF-2	EF-1α	GGARGTACCAGTSATCATGTT	O'Donnell *et al.,* 1998
EF-3	EF-1α	GTAAGGAGGASAAGACTCACC	O'Donnell *et al*., 2008
EF-22T	EF-1α	AGGAACCCTTACCGAGCTC	O'Donnell *et al*., 1998
MS1a	mtSSU	CAGCAGTCAAGAATATTAGTCAATG	White *et al*., 1990
MS2a	mtSSU	GCGGATTATCGAATTAAATAAAC	White *et al*., 1990

2.2.6 Phylogenetic analysis

In order to understand the genetic relationships and evolutionary history of the *Fof* isolates, phylogenetic trees were constructed using *F. oxysporum* EF-1α nucleotide sequences and mtSSU nucleotide sequences. Consensus sequences for EF-1α and mtSSU *F. oxysporum* isolates were produced using Geneious V7.1 (Biomatters Ltd, 2013). Consensus sequences of *F. oxysporum* isolates were aligned and edited using ClustalW in Geneious. For the EF-1α alignment, 26 *F. oxysporum* isolates were used. Two sequences of EF-1α from *Fof* isolates downloaded from GenBank were included as comparisons, Maff744009 from Japan and KJ776745.1 from Turkey, and a sequence of EF-1α *F. oxysporum* f. sp. *verticillioides (Fov)* (KF466424.1) (Genbank) was used to root the data set. For the mtSSU alignment, 25 *F. oxysporum* isolates were used (Table 2.1). Difficulties producing DNA products for isolate N9103 for mtSSU sequencing meant this isolate was excluded from the mtSSU phylogenetic analysis.

The trees were inferred using the Maximum Likelihood (ML) method based on the General Time Reversible model (Nei and Kumar, 2000). The initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Bootstrap analyses were made with 1,000 replications. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2416 for EF-1α, and parameter= 0.5082 for mtSSU). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites for EF-

1α, and 53.9565% sites for mtSSU). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

To assess the incongruence between EF-1α and mtSSU data sets and to determine whether the data sets would converge toward the same phylogenetic tree an incongruence length difference test (Farris *et al.,* 1994) was performed using PAUP version 4.0.

2.3 Results

2.3.1 Pathogenicity testing

Nine *F. oxysporum* isolates were tested in root dip inoculations to determine their virulence on the susceptible cultivar Kabarla. The rate and degree of symptom expression in plants varied among isolates. Plant symptoms included: chlorosis and necrosis of the leaves, lesions on the petioles, the wilting and collapse of petioles and leaves, and eventual plant death (Figure 2.2a). The earliest wilting symptoms occurred 3 weeks post-inoculation with plants inoculated with isolates N13581, N15309, N15457, N17337 and N18462. Two plants inoculated with N15457 did not show wilt symptom until 6 weeks after inoculation. Isolates that caused disease symptoms of Fusarium wilt and had a mean disease severity ratings >2 were designated as *Fof*. The majority of plants inoculated with N16004, N18419 and N18421 did not show any symptoms at the end of the evaluation period (at 8 weeks post-inoculation). Most plants inoculated with isolates N18419 and N18421 and the controls remained healthy and upright (Figure 2.2b), however, three plants had disease ratings of either 1 or 2, indicating either a slight wilt or stunting. These ratings (symptoms) were not considered severe enough to represent *Fof* but were still documented.

The difference in pathogenicity among the nine isolates, based on the disease severity index, was used to classify isolates as either pathogenic or non-pathogenic. Six of the isolates tested (N17337, N13581, N15309, N18462, N15457 and N18582) caused Fusarium wilt disease on strawberry. These isolates were classified as pathogenic and determined to be of the *forma specialis* '*fragariae*'. The remaining three isolates (N16004, N18419 and N18421) resulted in typically healthy plants, with clean crowns, were comparable to non-inoculated control plants, and no isolates were recovered from sampled crowns. As there was no significant difference between the controls, these isolates were regarded as non-pathogenic to strawberry and therefore not of the *forma specialis* '*fragariae*'.

(a) 

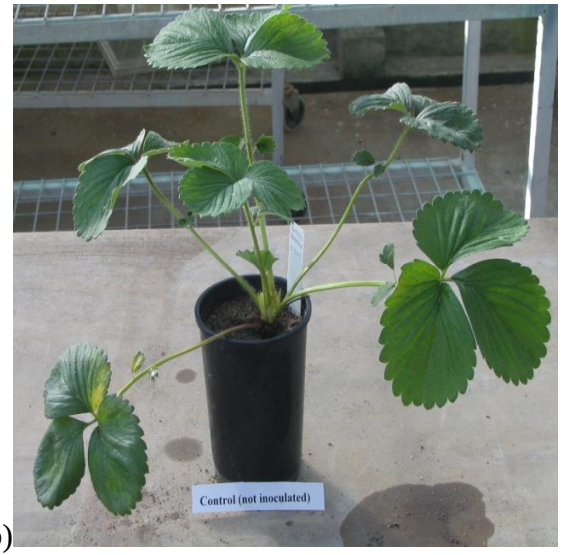
(b) 

Figure 2.2 Five weeks after inoculation, symptoms of collapsed and desiccated plants were evident on the plants inoculated with isolate N17337 (a) compared to the non-inoculated control (b) showing no symptoms.

To determine differences in virulence among the *F. oxysporum* isolates, the disease severity ratings, taken from all plants at 8 weeks post-inoculation, were analysed using ANOVA (average SE of difference equal to 0.93). Plants inoculated with isolates N17337 and N13581 scored the highest disease severity ratings (Table 2.3). There were significant effects ($P<0.05$) of pathogenicity. N13581, N17337 and N18462 were significantly more virulent of all isolates tested to be pathogenic. The least virulent isolates of those tested to be pathogenic were N15457 and N18582. All non-inoculated control plants were healthy for most of the experiment; however, one plant had a slight wilt at the end of the assessment. There was no significant difference between the controls and inoculations with isolates N18421, N16004 and N18419.

2.3.1.1 Crown sampling for Fof recovery

The level of discolouration in the vascular tissues of the crown was consistent with that of foliar disease severity. Typically, plants showing symptoms exhibited vascular discolouration and rots, while those showing no symptoms had clean crowns and non-discoloured vascular tissues. Diseased crowns showed reddish-brown to dark brown colouration of the vascular tissue of the crown and some rot or discolouration of the pith within the crown. Crown isolations carried out on symptomatic plants produced significant fungal colonies and were identified by morphological characteristics. Typically these

included the characteristic light purple colour of colonies and hyaline micro/macro conidia when plated onto PDA. *F. oxysporum* was re-isolated from all crown samples tested with the exception of plants inoculated with N16004, N18419, and N18421, and one plant inoculated with N18582. All the control crowns were symptom free and clean with the exception of one plant.

F. oxysporum was harvested from the crown of one of the control plants. This was believed to be due to incorporated controls amongst isolate-tested plants in the completely randomised experimental design. The close spacing of pots (25 to 30 cm) may have contributed to contamination from neighbouring pots. To limit contamination of control plants in pathogenicity testing and screenings, placing control plants both within inoculated plants and on separate tables at least one metre apart from inoculated plants was employed in the following thesis chapters.

2.3.1.2 Confirmation of pathogenicity tests

To confirm that the re-isolations, obtained from infected strawberry plants from the pathogenicity experiment, were pathogenic strains of *F. oxysporum* and to satisfy Koch's postulates, the re-isolates underwent further pathogenicity tests. These isolates included N13581, N15309, N15457, N17337, and N18582, and were given accession numbers N13581a, N15309a, N15457a, N17337a, and N18582a, respectively. At 6 weeks post-inoculation all plants inoculated were showing disease symptoms (Figure 2.3). There was no significant difference (average SE of difference equal to 0.81) in pathogenicity to strawberry between isolates N15309a, N13581a and N17337a. However there was a significant difference ($P<0.05$) between these isolates and the isolates N15457a, N18582a as well as the non-inoculated controls (Table 2.3 and 2.4 and Figure 2.3).

2.3.1.3 Sampling of re-isolation experiment

Crown isolations carried out on symptomatic plants produced significant fungal colonies, and were identified as *Fof* by colony morphological characteristics and their pathogenicity to strawberry. Crown and morphological symptoms were typical of those described in section 2.3.1.1. *F. oxysporum* was isolated from most of the crowns sampled, with the exception of the non-inoculated control plants. All the control crowns were symptom and disease free.

Table 2 3 Disease severity visual ratings produced by *F. oxysporum* isolates, taken at 8 weeks post-inoculation from pathogenicity experiment.

Isolate	Primary Disease Rating[z] (mean)(n=5)	Re-isolate code	Disease Rating[z] following re-isolation (mean)(n=6)	Pathogenicity rating[y]
n/a	n/a	**N17337**(comparison)	8.17_{d}	Pathogenic
N18421	0.6_{a}	**n/a**	n/a	Non-pathogenic
N16004	1.4_{a}	**n/a**	n/a	Non-pathogenic
N18419	1.4_{a}	**n/a**	n/a	Non-pathogenic
N18582	2.0_{ab}	**N18582a**	4.00_{b}	Pathogenic
N15457	3.4_{b}	**N15457a**	5.67_{c}	Pathogenic
N15309	7.2_{c}	**N15309a**	9.00_{d}	Pathogenic
N18462	7.8_{cd}	**n/a**	n/a	Pathogenic
N17337	9.2_{d}	**N17337a**	9.33_{d}	Pathogenic
N13581	9.6_{d}	**N13581a**	9.00_{d}	Pathogenic

Means with same subscript are not significantly different at *P*=0.05. LSD = 1.8

[z]Isolates were rated on a scale of 0-10, with 0=healthy and 10 = dead

[Y] Isolates were considered pathogenic if plants had a mean disease severity rating >2

(a). N17337 (comparative standard)

(b). N15309a

(c). 17337a

(d). N18582a

(e). N15457a

(f). N13581a

Figure 2.3 A second pathogenicity experiment (section 2.3.1.2) tested five *F. oxysporum* isolates, re-isolated from diseased crowns from the pathogenicity test, on 'Kabarla' plants (n=6). Isolate N17337 was used as a comparative. Photographs above were taken at 6 weeks post-inoculation and show inoculated plants on the left and control plants (non-inoculated) on the right.

2.3.2 Vegetative compatibility grouping of *Fusarium oxysporum* isolates

Nitrate utilization mutants, were generated from 25 isolates when cultured on MM containing potassium chlorate. These mutants were determined by sectors unable to utilize nitrate and identified by sparse growth, with no aerial mycelium (Figure 2.1b). Three to 25 mutants were generated from each isolate tested. Physiological phenotypes were determined by growing mutants on media containing three different nitrogen sources. Typically *nit* 1 mutants were produced, followed by *nit* 3 and then Nit M. All isolate sectors produced at least one *nit* 3 or Nit M to be used as testers. All combinations of isolates were paired on a MM medium with nitrate as the only nitrogen source to determine VCG. Samples paired were rated as belonging to the same VCG by a line of dense white aerial mycelium (heterokaryon formation) where the two isolates merge.

All mutants of an individual isolate were compatible with each other with the exception of isolates N9054, N9055, N9551, N10010, N10226, and N18582, and these were designated as self-incompatible. Four isolates from the Sunshine Coast region in Qld (i.e., N13581, N15309, N15457, and N15915) formed prototrophic heterokaryons and were grouped into the same VCG, designated 'VCGa'. Similarly, the four isolates sampled from WA (N16239, N16240, N17337, and N18462) produced well-formed heterokaryons and were designated 'VCGb'. The remaining isolates were considered single-member VCGs as they were unable to form a heterokaryon with another strain (Appendix 2.1).

2.3.3 Sequence and phylogenetic analysis

Following alignment of sequences, consensus sequences for each isolate were produced at 678 bp for EF-1α and 764 bp for mtSSU. The incongruence length difference test indicated that data sets for EF-1α and mtSSU were significantly different ($P<0.01$) and therefore could not be combined. These discrepancies meant converging toward a single phylogenetic tree would lead to conflicting conclusions, therefore the data sets were analysed separately.

2.3.3.1 EF-1α phylogenetic analysis

Phylogenic analysis of EF-1α sequences of the *F. oxysporum* isolates showed 29 isolates grouped into 10 lineages. Twenty-four isolates grouped into three clades (Figure 2.4). Clade 1 to 3 could be further divided into five lineages (i to v), representing 83% of

the isolates from regions within southeast Queensland, New South Wales, and Japan (Maff744009), all assumed therefore to share a common ancestor. Isolates from lineage (ii), were all closely related and occurred with highest frequency, representing 34% of all isolates assessed. Although there was no genetic variation among sequences in lineages (ii), it included isolates that grouped into VCGa as well as five single group isolates. Similarly, there was no genetic variation detected among the four sequences in lineage (i) all of which were from WA and comprised VCGb. The lineages (iii) to (v) comprised isolates of single member VCGs, and *Fof*-KJ776745.1 (untested). Results from the EF-1α sequence data set indicate that isolates SA126 and N18437 from lineage (vi) and (vii) were genetically dissimilar, and separated from the isolates in lineages (i) to (v) and outgroups (viii to x). The outgroup *Foz*-BRIP39298 (*F. zingiberi*), was separated from the *Fof* isolates, the only exception being N16004 which was even more distantly related from the other *Fof* isolates than *Foz*-BRIP39298. The results suggest that isolate N16004 was non-typical.

2.3.3.2 mtSSU phylogenetic analysis

Phyogenic analysis of the mtSSU sequences of the *F. oxysporum* isolates indicated that they clustered into four lineages ‘a’ to ‘d’ (Figure 2.5). Twenty-one isolates, representing 84% of the isolates, were grouped in one lineage ‘a’. There was no genetic variation detected among the sequences in lineage (a), except for isolate sequence N9551. Lineage ‘a’ comprised VCGa and VCGb. SA126 and Foz-BRIP39298 shared lineage ‘b’, while N18437 and 9055 separated in individual lineages ‘c’ and ‘d’. The isolates SA126 and N18437 separated into distinct lineages ‘b’ and ‘c’. Results from the mtSSU sequence data set suggest that there are at least two independent origins.

An anomaly in the mtSSU data set was due to the sequence of N9055. This sequence had a 9 bp insertion, causing separation as graphically represented by branch length in Figure 2.5, which was not seen in the EF-1α tree. Other differences between the EF-1α and mtSSU data sets included the grouping of SA126 with the *Foz* isolate BRIP39298 in the mtSSU phylogeny while clustering separately in EF-1α. As discussed above, the EF-1α *Fof* sequences clustered into seven lineages (Figure 2.4), while the mtSSU sequences only identified four lineages (Figure 2.5). Additionally, the isolates SA126 and N18437 from lineage ‘b’ and ‘c’ in the mtSSU analysis were both genetically dissimilar to each other, separating into distinct lineages, but in EF-1α analysis shared a common

lineage (Figure 2.5). The mtSSU analyses, inferred from the branching topology, did not show any correlation between the distribution of isolates and the geographic origin.

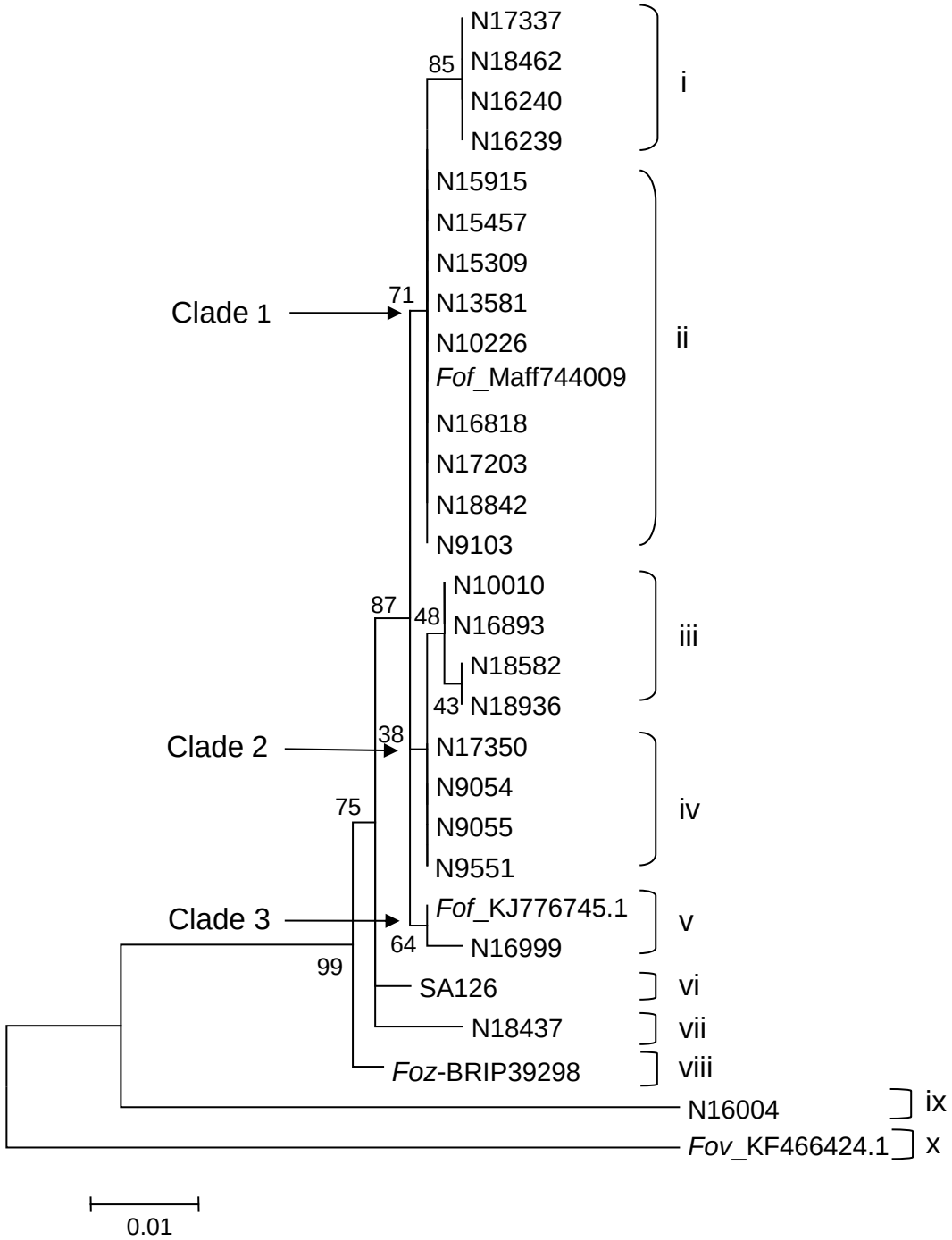


Figure 2.4 The phylogenetic tree of 29 EF-1α nucleotide sequences rooted with *F. oxysporum* f. sp. *verticillioides* (KF466424.1) was inferred by the ML method based on the General Time Reversible model (Nei and Kumar, 2000). *Foz*-BRIP39298 and

N16004 were also included as outgroups. Branch lengths are measured in the number of substitutions per site. Numbers at the nodes represent bootstrap values from 1,000 replications. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013). Ten clonal lineages are identified by (i to x) and the three clades are indicated by arrows next to the corresponding interior branches.

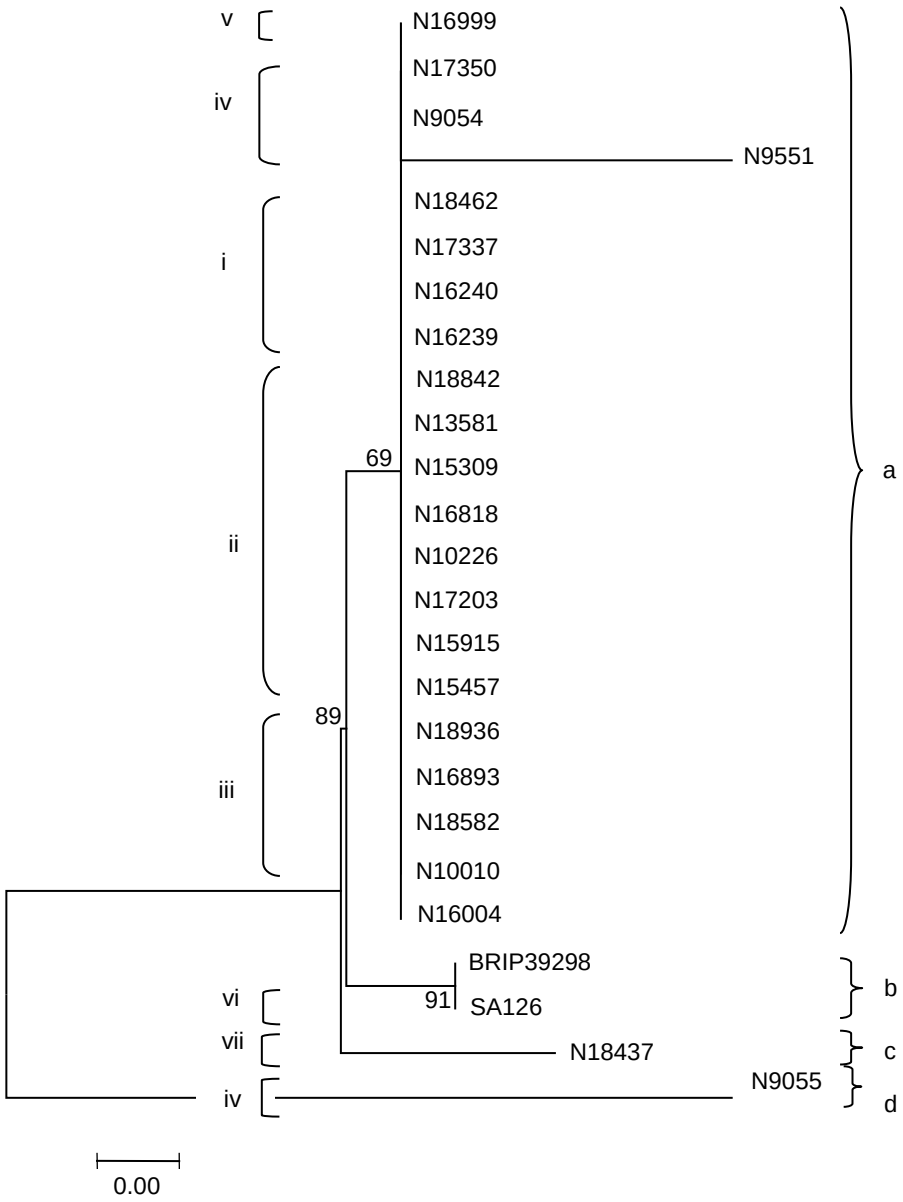


Figure 2.5 The phylogenetic tree of 25 mtSSU nucleotide sequences, inferred by the ML method based on the General Time Reversible model (Nei and Kumar, 2000). Branch lengths are measured in the number of substitutions per site. Numbers at the nodes represent bootstrap values from 1,000 replications. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013). Lineages from the EF-1α sequences (i to x) are indicated next to brackets on left of tree while lineages for mtSSU are indicated on right ‘a’ to ‘d’.

2.4 Discussion

Understanding pathogenicity of different *Fof* isolates is important for strawberry breeding programs to ensure strawberry genotypes are challenged by virulent strains (i.e., pathogenic and commercially relevant), and superior strawberry genotypes can be selected. In this study an in depth assessment of pathogenicity of isolates from the major strawberry production areas in Qld and WA, showed disease response was significantly different. These findings agree with Fang *et al.* (2011) who also found disease severity was significantly affected by isolates of *F. oxysporum* (eight isolates) acquired from strawberry farms in WA. The mean rating for disease severity ranged from nil (no symptoms), to moderate (wilting and some plant death), to severe (the death or pending death of most plants), indicating heterogeneous populations of *Fof*.

Pathogenicity tests undertaken in this study identified, four virulent isolates; N13581, N15309, N17337, and N18462. These isolates were chosen for evaluations of cultivar susceptibility to Fusarium wilt disease and for screening for *Fof* resistance. Use of a *Fof* strain with demonstrated pathogenicity is imperative for reliable assessment of *Fof* susceptibility. Although the WA strain N17337 was severely virulent, use of this strain in screening tests in Qld was rejected due to the possibility of accidental release into the environment.

Twenty-six isolates were also categorised using complementation analyses (complementation testing of mutants) with the exception of the publicly available. Assignment of isolates into VCGs was difficult with 19 isolates belonging to single-member VCGs. Five isolates were identified as self-incompatible, lacking heterokaryon formation, even though several attempts with complementation tests were performed. This may have been due to problems generating appropriate mutants, or the isolates were not a pathogenic strain of *F. oxysporum* and/or genetically dissimilar. However, two distinct VCGs were identified among the 25 isolates tested and correlated with differences in geographic origin; N13581, N15309, N15457, and N15915, all of which originated from southeast Qld, grouped into VCGa, and the isolates N16239, N16240, N17337, and N18462 from the Perth district in WA, grouped into VCGb.

Genetic analyses were used to further characterise isolates. Phylogenetic analyses based on EF-1α sequences showed variability among isolates associated with different regions. For the majority of isolates, obtained from within southeast Queensland, there was moderate genotypic diversity. In contrast, all of the isolates from WA were of the

same genotype and VCG. This limited diversity could be due to the isolates sampled from only a small area in the Perth district, and a wider range of isolates may need to be tested to obtain a better representation of genetic diversity in WA.

The EF-1α sequences of *Fof* isolates within clade 1 (Figure 2.4) were determined to be closely related and represented individuals from Qld, New South Wales and WA, but also from Japan (Maff744009). The possibility that this genotype was introduced into Australia from Asia or vice versa remains to be clarified. The geographical spread of this genotype, over three states of Australia, and in Japan, is suggestive of the adaptability of *Fof* to different environments and shows a potential risk if spread to the colder regions in southern Australia, especially if susceptible cultivars are grown. Sequences within clade 2 were also closely related; however, there was sequence variation within the lineages. *Fof* sequences within clade 3 were genetically dissimilar, but shared a recent common ancestor with clades 1 and 2.

Results of the EF-1α analysis and pathogenicity testing of isolates indicate that isolates belonging to clades 1 to 3 (lineages 1 to v) belong in the *forma specialis* '*fragariae*'. The remaining divergent lineages - SA126 and N18437 (lineages vi and vii), were concluded to be genetically dissimilar. SA126 was identified as of the *forma specialis 'fragariae'* as this isolate was pathogenic to strawberry in the pathogenicity test described in Chapter 3 (section 3.2.2). The pathogenicity status of N18437 remains to be clarified. The phylogeny of mtSSU and to some extent EF-1α implies that there are at least two evolutionary origins of *Fof*, i.e., lineages 'a' and 'b' in mtSSU phylogeny lineages, and lineages (i) to (v) and lineage (vi) in EF-1α, indicating the *forma specialis* is not of monophyletic origin. The isolate N16004 was determined not of the *forma specialis* '*fragariae'.*

The decision to use the EF-1α and mtSSU gene regions in this study was based on the common use and results obtained from studies of genetic similarities among *formae speciales* of *F. oxysporum* (O'Donnell *et al*., 1998; White *et al.*, 1990; Baayen *et al.*, 2001; Skovgaard *et al.,* 2001; O'Donnell *et al.*, 2004; Bogale *et al.,* 2006; Rahjoo *et al*. 2008; Fourie *et al.,* 2009). Although relationships within *Fof* were clearly defined by EF-1α analysis and suggest more divergent evolution of EF-1α sequences in different isolates of *Fof*, the few sequence differences revealed from mtSSU analysis was generally insufficient to resolve interspecific and intraspecific genetic variation among the *Fof* isolates. O'Donnell et al. (1998) found that better resolution with EF-1α gene than the

mtSSU, possesses more phylogenetic information in *F. oxysporum*. However, the mtSSU analysis did provide additional phylogenetic information, suggesting independent evolution of some isolates.

Collectively, the results from pathogenicity testing, VCG and EF-1α analysis indicate a diverse range of pathogenicity and genotypes occurring in *F. oxysporum* isolates obtained from Australian strawberry farms, and these include both pathogenic and non-pathogenic strains. Isolate N16004 grouped with pathogenic isolates in the mtSSU phylogeny but based on EF-1α N16004 belongs to a separate lineage more closely aligned to *Fov*. Additionally, this isolate is non-pathogenic to strawberry and showed dissimilar colony morphology to the *Fof* isolates. This implies the mtSSU may not adequately distinguish the genetic variation at the interspecific level. This remains to be clarified and warrants further investigation.

Nagarajan *et al*. (2004) found a relatively high correlation among RAPD and VCG, and pathogenicity among isolates of *Fof*. Fourie *et al.* (2009) found isolates of *Foc* associated with the same VCG had identical EF-1α and mtSSU sequences, and that they clustered together irrespective of their geographic origin. The results of the present study involving EF-1α, VCG, and pathogenicity were consistent with these findings. The Isolates tested in the pathogenicity tests that were pathogenic; N17330, N18462 from Western Australia and N13581, N15309, N15457 and N18582 from Queensland; correlated with lineages (i) and (ii) of the EF-1α phylogenetic tree and associated with the same VCG. The non-pathogenic isolate N16004 belonged to a separate linage from those pathogenic. The results presented from VCG testing indicate that vegetative compatibility, although useful by implying close genetic associations, is alone insufficient for the characterising of pathogenic *F. oxysporum* isolates as several *Fof* isolates were single member VCGs. VCG in combination with pathogenicity testing and phylogenetic analyses is, however, useful for identifying genetic differences among *formae speciales* of *F. oxysporum* and within the *forma specialis 'fragariae'*.

Although the isolates profiled represent only a small portion of those in the strawberry production areas of Australia and focuses mainly on Qld, some isolates were highly virulent *Fof* strains affecting strawberry. The results presented suggest that there is a moderate level of genotype diversity in the *Fof* isolates profiled. This research will be useful to assist in the detection and monitoring of pathogenic strains in strawberry production fields, provide strawberry growers with information to choose cultivars, and for

resistance breeding. However, this study illustrates limitations of phylogenetic analyses using partial gene regions, which could be overcome in future studies by employing next generation sequencing to obtain whole genome sequences. Such an inclusive data base of *Fof* sequences and comparative genomic analysis should enable more insight, leading to the identification of pathogenicity genes.

3.0 Chapter 3 Development of a glasshouse bioassay suitable for evaluating Fusarium wilt resistance in strawberry

3.1 Introduction

To identify sources of plant genetic resistance, an effective and reliable bioassay is required for screening large numbers of strawberry genotypes to identify those with desirable levels of resistance. Successful and accurate disease development is fundamental to the screening process, and dependent on pathogen virulence, correct inoculum levels and favourable environmental conditions.

Various screening methods have been used to evaluate strawberry germplasm for resistance to *Fof.* These have included evaluation in infested soils in open fields (Toyoda *et al.*, 1991; Hutton *et al.*, 2006; Takahashi *et al.*, 2003); inoculation of plants with *Fof* conidial suspension under glasshouse conditions, either at early seedling stage (Takahashi *et al.,* 2003) or at mature plant stage (Fang *et al.,* 2012); and evaluation of resistance in strawberry cultivars grown in water culture inoculated with *Fof* (Kuroda and Tomikawa, 2001).

An effective bioassay is important for the inoculation of plants in resistance breeding. This study was initiated to compare and evaluate *Fof* inoculation methods. Two conidial-suspension methods (root-dip and injection), including three conidial concentrations within the root dip method; and two substrate inoculation methods (ryegrass and millet seed) were tested under glasshouse conditions for their use and efficacy in inoculations with subtropical strawberry germplasm.

3.2 Materials and methods

3.2.1 Plant preparation

Strawberry plants of the *Fof* susceptible cultivar Kabarla, as determined by Hutton and Gomez (2006), were planted into 100mm x 140mm pots containing steam-sterilised potting mix composed of double washed river sand and coir in a 1:1 ratio by volume and a pre-mixed fertiliser of (g/L) 5.1 nitrogen, 7.2 phosphorus, 4.6 potassium, 60.4 calcium, 0.09 copper, 0.06 iron, 0.32 magnesium, and 0.15 zinc. The potted plants were watered and fertilised as required until established.

3.2.2 Isolates

Fof isolates N13581 and N15309 (Table 3.1) were harvested from the crowns of symptomatic strawberry plants of Fusarium wilt from southeast Queensland. Isolates were stored as single-hyphal tip cultures on filter paper and housed at Maroochy

Research Facility (MRF), Nambour, Qld. The isolates were identified as virulent strains of *Fof* based on pathogenicity testing described in Chapter 2 (section 2.3.2), and belonged to the same vegetative compatibility group (VCG).

Table 3.1 *Fof* isolates used in inoculation experiments.

Isolate	Year collected	Origin in Australia	Cultivar host	VCG[z]
N13581	2002	Palmwoods, Qld	Kabarla	A
N15309	2005	Wamuran, Qld	Camarosa	A

[z] As defined in chapter 2

3.2.3 Conidial-suspension inoculum preparation

The isolates were aseptically plated onto streptomycin amended 1/4 strength potato dextrose agar (SPDA) and incubated at 24°C for 1 week, after which they were sub-cultured onto plates of 1/4 strength SPDA and incubated at 24ºC for 3 weeks. The spores were collected from culture plates following addition of sterile deionised water and rubbing the agar surface with a glass spreader. The spore suspension was then filtered through four layers of cheesecloth. The conidial concentration was determined using a haemocytometer and adjusted to 1×10^6 conidia/mL.

3.2.4 Substrate inoculum and pot preparation

Ryegrass (*Lolium* sp.) and millet (*Echinochloa esculenta*) seed were prepared following techniques modified from those described by El-Tarabily *et al*. (1997), Smith *et al.* (2008), and Rames *et al*. (2009). The seeds were soaked in tap water for several hours and rinsed in distilled water, after which the excess water was drained. Seeds were then transferred into 500mL plastic tubs containing sterile distilled water at a volume sufficient to imbibe seed, i.e., approximately 120mL and 50mL for ryegrass and millet, respectively. The tubs were then autoclaved on three consecutive days, for 20 mins at 121°C each time.

The tubs of both ryegrass and millet seeds were inoculated with eight plugs of fungal mycelium, each with a plug diameter of 6mm, taken from marginal colonies of isolates grown on 1/4 strength SPDA plates. Tubs were then incubated at 27°C until visible colonisation of *Fof* throughout the seeds was detected, which took approximately 2 weeks (Figure 3.1). During this time, the tubs were shaken several times to distribute the

Fof evenly throughout the seed. Viability of the pathogen was confirmed by observing mycelium growth from colonised seed when grown on 1/4 strength SPDA. Tubs of each seed type without the addition of the inoculum served as controls.

Figure 3.1 Ryegrass seed colonised with *Fof*.

Eight weeks prior to plants being inoculated, pots containing the strawberry plants to be treated with ryegrass and millet seed where prepared as follows. Two 100mm lengths of sterilised 12mm diameter poly-pipe tubes were vertically inserted opposite one another, into the soil at the internal edge of each pot. These tubes served as a conduit for the inoculated substrate to be delivered directly to the root zone at the time of inoculation. The 8 weeks between insertion of tubes and inoculation allowed any root damage by the pot preparation process to heal and function normally.

3.2.5 Experiment 1: Substrate comparison

This experiment was conducted in July 2010 at MRF. Using the isolate N13581 only, two substrate inoculation methods, inoculated millet seed and ryegrass seed, were tested on the strawberry cultivar Kabarla. Four plants were used per substrate treatment and four plants per control (non-inoculated seed) treatment.

Prior to inoculation, the two poly-pipe tubes were removed from pots containing plants and approximately 15g of inoculated seed poured into each of the two empty spaces made by the tubing in each pot. The remaining void was filled with extra soil mix. Approximately 10mm of sterile gravel was placed on top of the soil mix around the plant to prevent inoculum exposure and contamination from splash. To prevent cross contamination the control plants were treated first, using sterile seed (non-inoculated) only.

Inoculated plants were placed 250mm apart on a heated bench at 28°C with natural lighting in a glasshouse. The control plants were separated from inoculated plants by at least a distance of 1m to avoid contamination. Plants were watered daily for the first 2 weeks after inoculation and three times per week thereafter.

3.2.6 Experiment 2: Inoculation method comparison

Four inoculation methods: (1) root dip, (2) injection, (3) colonised ryegrass seed, and (4) colonised millet seed were tested on cultivar Kabarla, November 2011 at MRF. Ten plants were used per inoculum treatment and six plants per control (non-inoculated seed) treatment. For treatments 1 and 2, prior to inoculation, the conidial suspensions of N13581 and N15309 (mainly microconidia and macroconidia with some chlamydospores) were combined in equal amounts to a final concentration of 1.0×10^6 conidia/mL.

1. Root dip: Plants were removed from their pots and the roots and crown washed free of soil and debris, after which they were immersed in the *Fof* spore suspension for 10mins. To prevent cross contamination the control plants (non-inoculated) were treated first by immersing in sterile water only. The plants were then potted into 100mm x 140mm pots using potting mix described previously and approximately 10mm of sterile gravel added on top of the soil mix around the plant to prevent contamination from splash.

2. Injection: Plants were injected, penetrating approximately 1 to 2 mm in two places at the base of the crown with 0.1mL of *Fof* conidial suspension at a concentration of 1×10^6 conidia/mL, using a 1.0 mL syringe (Terumo™) with a BD (PrecisionGlide™) needle, 27G ½ (0.4mm x 13 mm). Control plants were injected with sterile water only.

3 and 4. Substrate: As in experiment 1, prior to inoculation, the two poly-pipe tubes were removed from pots containing plants and approximately 15g of inoculated seed was poured into the two empty spaces and was filled with extra soil mix. Sterile gravel was

placed on top of the soil. Control plants were given the same treatment, but using sterile seed only.

Inoculated plants from all inoculation treatments were placed 250mm apart on heated benches at 28°C, with four replicates (benches) in the glasshouse under natural lighting. The control plants were placed on a separate bench by a distance of 1m to avoid contamination. Plants were watered daily for the first 2 weeks after inoculation and 3 times per week thereafter.

3.2.7 Experiment 3: Concentration of inoculum

The *Fof* isolate N13581 was tested at three spore concentrations on the cultivar Kabarla, in July 2010 at MRF. Inoculum preparation was as described previously, except that the 3-week subculture was incubated at 27°C. The resulting conidial suspension was made up into three concentrations, 2×10^6, 1×10^6, and 1×10^5, by diluting with sterile water. Plants were inoculated and managed as in the root dip method in experiment 2 (section 4.2.5.2) but with six 'Kabarla' plants.

After inoculation, the pots were placed 250mm apart on a heated bench at 28°C. The control plants (no *Fof* inoculum) were separated from inoculated plants by approximately 1m to avoid contamination.

3.2.8 Disease severity assessment

In all experiments, disease development was monitored weekly on the individual plants and visual disease severity ratings taken after the first symptoms appeared. Severity of foliar symptoms were assessed on a 0 to 10 disease visual index (Hutton and Gomez, 2006) as described in Chapter 2. A mean disease severity score for each treatment was calculated across replicates.

3.2.9 Crown sampling for *Fof* recovery

Symptomatic plants, and a sample of healthy plants (if available) representing each inoculation procedure in all experiments were sampled. The crowns were washed clean and surface sterilised using sodium hypochlorite and rinsed three times in sterile water. Crowns were cut in cross sections and discoloured pieces of crown plated onto 1/4 strength SPDA and assessed following incubation at 24°C for 1 week.

3.2.10 Statistical analysis

All statistical analyses were performed using Genstat (version 11.1) (VSN International Ltd.). Treatments were compared using disease severity rating means analysed through Analysis of Variance (ANOVA) and Fisher's protected least significant difference test ($P<0.05$).

3.3 Results

3.3.1 Experiment 1

At 10 weeks post-inoculation, plants from both seed treatments were either dead or showing symptoms of Fusarium wilt with the exception of one Kabarla × millet inoculation (Table 3.2). Symptoms included: chlorosis and necrosis of the leaves, lesions on the petioles, and the wilting and collapse of petioles and leaves, increasing in severity in the mature leaves.

Only the mean visual ratings from week ten were analysed (Table 3.2). The difference between types of seed used for inoculum was not significant. Both inoculum types, whether millet or ryegrass seed based, lead to severe symptoms and plant death, and all sampled crowns showed internal *Fof* symptoms, which included discolouration of the vascular tissues, crown pith and crown rots. *Fof* was re-isolated from sampled crowns of symptomatic plants from both *Fof* inoculated seed treatments. All control plants were free of symptoms and sampled crowns healthy and free of vascular discolouration

Table 3.2 Assessment and incidence of Fusarium wilt on 'Kabarla' using two substrate treatments (ryegrass seed and millet).

Treatment	Number of plants showing symptoms (n=4)	Mean disease ratings [z]	*Fof* re-isolated from crown
Millet x Kabarla	3	7.0 $_{a}$	4/4
Ryegrass x Kabarla	4	8.7 $_{a}$	4/4

[z] Treatment means: 0 = healthy plants showing no symptoms and 10 = total death of plant. Means with same subscript are not significantly different at *P*= 0.050

3.3.2 Experiment 2

Plants inoculated by root dip method were the first to show symptoms at 4 weeks post-inoculation. Symptom development was delayed until 6 weeks post-inoculation in plants from both seed inoculation methods. Symptoms included those previously described in experiment 1. The majority of plants in all inoculation methods, except by injection of spore suspension, were either dead or showing symptoms of Fusarium wilt by 10 weeks post-inoculation. Two symptomless plants occurred in the root dip method and these were regarded as escapees, as the majority of plants in this treatment showed severe wilting symptoms and/or death.

The design was treated as a 2 × 4 factorial with two levels (control and inoculated) x four levels of treatment (Trt) (dip, injected, millet and ryegrass). Four disease rating (assessment) dates were analysed: weeks 7 to 10 post-inoculation. At 7 weeks post-inoculation (rating 1), there was a significant difference in disease rating between control and inoculated plants, although there were no significant difference among the four treatments. At 8 weeks post-inoculation (rating 2), there was a significant inoculation treatment effect. The highest disease severity rating was found for the ryegrass inoculated treatment (Table 3.3). At 9 and 10 weeks post-inoculation (ratings 3 and 4), the disease rating on inoculated ryegrass treatment was again significantly higher than in the other treatments. The disease rating for the injected treatment was significantly lower than the inoculated ryegrass, millet, and dip treatments and similar to the control treatments.

Fof was re-isolated from sampled crowns of symptomatic plants from root dip and both seed inoculum treatments, and showed typical *Fof* morphologies, e.g., the characteristic light purple colour of colonies and hyaline micro and macro conidia. The crowns of plants sampled from the injected treatments were clean as was 1 crown sampled from the root dip method. One plant from the injected control treatment, at week 10 post-inoculation, showed strong symptoms of Fusarium wilt. When sampled, *F. oxysporum* was re-isolated from the crown. This plant was determined to have been contaminated, the inoculation source and method of infection is unknown.

Figure 3.2 Strawberry plants showing wilt symptoms (front row) were inoculated using ryegrass seed colonised with *Fof*. Healthy (control non-inoculated) plants are in the rear.

Table 3.3 Mean disease severity ratings of *Fof* inoculated strawberry plants for eight levels for control and treatment, at three rating times (8, 9 and 10 weeks) post-inoculation.

	Treatment	7 weeks	8 weeks	9 weeks	10 weeks
Trt	Ryegrass	2.2_a	5.50_a	8.40_a	9.80_a
Trt	Dip	0.8_a	1.30_b	3.00_b	4.10_b
Trt	Millet	0.6_a	0.90_b	1.90_{bc}	3.10_{bc}
Trt	Injected	0.1_a	0.00_b	0.20_c	0.40_d
Control	Ryegrass	0.0_b	0.00_b	0.00_c	0.00_d
Control	Dip	0.0_b	0.00_b	0.00_c	0.00_d
Control	Millet	0.0_b	0.00_b	0.00_c	0.00_d
Control	Injected	0.0_b	0.00_b	0.83_{bc}	1.50_{cd}

Means with same subscript are not significantly different at $P = 0.05$

3.3.3 Experiment 3

At 11 weeks post-inoculation, 17 of the 18 *Fof* inoculated plants were showing symptoms of Fusarium wilt. Disease severity ratings in inoculum concentration treatments were similar to each other, ranging from 7.3 to 9.3, but all were higher than the control. All control plants were healthy and free of symptoms, including internal discolouration. Only the visual disease ratings from week 11 were analysed, showing no significant difference between the different inoculum concentration treatments (Table 3.4).

Fof was re-isolated from sampled crowns of infected plants from all inoculum concentration treatments. These colonies showed typical *Fof* morphologies as described previously.

Table 3.4 Plants showing symptoms and mean disease severity ratings, at 11 weeks post-inoculation of plants inoculated with three concentrations of *Fof* inoculum.

Treatments Spores/mL	**Plants showing symptoms (N=6)**	**Mean ratings**
1 x10^6	6	9.3_a
2 x10^6	6	7.3_a
1 x10^5	5	8.8_a
Control(sterile water)	0	0.0_b

Means with same subscript are not significantly different at $P = 0.05$

3.4 Discussion

This study investigated methods to optimise the delivery of *Fof* inoculum to strawberry plants roots and assessed their use and suitability to subtropical strawberry germplasm in Qld.

While the population size of experiment 1 was small (only four replicates per treatment), this experiment demonstrated the effectiveness and potential of the substrate method, and gave insight into other aspects relevant for screening, e.g., time and resources needed to undertake the screening process, damage to plant, and ease of use.

Fof inoculated millet and ryegrass seed incorporated in the potting mix, established effective plant infection, disease development, and plant death. The ryegrass and millet methods were easy to administer and required less resources and labour when compared to the root dip method. Glasshouse bioassays using an inoculated substrate for the purpose of host plant infection and disease development have proved effective for *F. oxysporum* species in other crops. Millet grain pre-colonised by *F. oxysporum* f. sp. *cubense* have been successfully used as a source of inoculum for the infection of banana (Smith *et al.*, 2008), and wheat seeds inoculated with *F. oxysporum* f. sp. *vasinfectum* has been used to infect cotton (Becerra Lopez-Lavalle *et al*., 2012). However, this study is the first time ryegrass and millet seed pre-colonised with *Fof* has been tested on strawberry.

The root dip method has commonly been practiced with many pathogens of strawberry for isolate pathogenicity and plant resistance evaluation tests (Noguchi *et al.*, 1994;

Freeman *et al.*, 2001; Takahashi *et al.*, 2003; Fang *et al.*, 2011). However, this method can be challenging, time consuming, and considered to be aggressive to plants (Smith *et al*., 2008; Becerra Lopez-Lavalle *et al*., 2012). There is a high probability of damaging roots, crowns, or leaves, and as a consequence influence subsequent plant and pathogen responses and disease infection. Additionally, there is the potential for escapees, which may result from either isolate loss of virulence, plant defences, or unfavourable environmental conditions. The probability of escapees from the root dip method was considered in this study. However, this method has performed more efficiently in other experiments described in this thesis and including experiment 3 (section 3.3.3). This inconsistency demonstrates the challenges associated with the root dip method.

Although symptom expression took longer following inoculation with infested ryegrass seed, this method had a significantly higher disease rating than all the other treatments (Table 3.3) and offers promise for use in large scale glasshouse inoculations of subtropical strawberry. Additional experiments are suggested to ensure the infection rates obtained occurs consistently across environments and cultivars. For inoculations of *Fof* using the root dip method, a concentration of either 1×10^6 or 1×10^5 conidia/mL is recommended for use in small scale inoculations.

From the results of this study, it was decided to use ryegrass seed as a substrate of *Fof* for the resistance screenings undertaken in section 5.0.

4.0 Chapter 4 Cultivar responses to *Fusarium oxysporum fragariae*

4.1 Introduction

Cultivar differences to *Fof* are an important aspect in resistance breeding and for the testing of pathogenic strains, so a range of genotypes can be tested to measure disease intensity. Knowledge of genotype differences allows the breeder to select genotypes appropriate for use in transferring the resistance trait, and gives growers options for disease management strategies, i.e., using disease tolerant or resistant cultivars.

Cultivar differences in strawberry in response to Fusarium wilt have been reported in field studies in Japan (Kuroda and Tomikawa, 2001; Mori *et al*., 2005) and Korea (Kim *et al.*, 1982). In California, cultivar differences were observed when outbreaks of *F. oxysporum* and *Macrophomina phaseolina* occurred in the major strawberry production areas. Plant losses and disease severity were greatest for the cultivars Camarosa and Albion (Koike, 2009), which are cultivars commonly grown in WA.

In Australia, attempts to differentiate cultivars in their responses to *Fof* have been limited. The first attempt to differentiate cultivar responses was conducted in Qld by Hutton and Gomez (2006), who tested cultivar responses to *Fof* in naturally infested fields located at Maroochy Research Facility, in Nambour. In this location they determined that the cultivar Kabarla was susceptible, while cultivars Festival, Rubygem and Sugarbaby were tolerant to *Fof*. From a survey conducted in WA by the Department of Agriculture and Food (2005 to 2006) following severe losses from wilt diseases suffered in the 2005 season, the cultivar Camarosa was reported to be susceptible to *Fof* (Golzar *et al*., 2007). Although resistant and susceptible cultivars have been identified, a wider range of specific Australian *Fof* isolates needs to be tested on a range of cultivars, and the interactions between cultivars and isolates investigated.

4.2 Materials and methods

4.2.1 Plant and isolate preparation

The isolates used in this study (Table 4.1) consist of six isolates from three diverse geographic areas of Australia. Previous studies (Chapter 2) indicated isolates N18462, N15306, and N13581 as virulent and N18582 as moderately virulent. The remaining two isolates were of untested pathogenicity. Isolates were stored as single-hyphal tip cultures on filter paper, and housed at Maroochy Research Facility, Nambour, Queensland.

Table 4.1 *Fof* isolates used to inoculate strawberry cultivars in cultivar response experiments.

Accession Number	Year collected	Origins	
N18462	2009	Western Australia	Wanneroo
N15309	2005	Queensland	Wamuran
N13581	2002	Queensland	Palmview
N18582	2009	Queensland	Stanthorpe
SA126	1981	South Australia	Adelaide Hills
SA127	1981	South Australia	Adelaide Hills

Strawberry plants obtained as bare-rooted runners from a certified (Plant Protection Amendment Regulation, No. 3, 2013, Qld.) runner nursery. The runners were planted into 100mm × 140mm pots containing steam-sterilised potting mix composed of double washed river sand and coir (by volume 1:1) with a pre-mixed fertiliser of (g/L) 5.1 nitrogen, 7.2 phosphorus, 4.6 potassium, 60.4 calcium, 0.09 copper, 0.06 iron, 0.32 magnesium, and 0.15 zinc. The potted plants were stored in a shade house, watered, and fertilised with Scotts Osmocote Plus (N14:P9:K15:Mg2 plus trace elements) slow release fertiliser and Yates Aquasol Soluble fertiliser (N23:P4:K18 plus trace elements) until required (approximately 6 months). Within a day prior to inoculation all dead or older leaves, runners, or flowers were removed from the plants.

Isolates of *Fof* were aseptically plated onto 1/4 strength potato dextrose agar amended with 50 ppm streptomycin sulphate (SPDA) and incubated at 27°C for 2 weeks. The isolates were subcultured onto 20 plates of 1/4 strength SPDA and incubated for 3 weeks. Spores were collected following addition of sterile deionised water and dislodged by rubbing the agar surface of culture plates with a glass spreader. The spore suspension was then filtered through four layers of cheesecloth. The conidial concentration was adjusted to 1.0×10^6 conidia/mL using a haemocytometer. The inoculum was used within 5 hours.

4.2.2 Experimental design

Three separate experiments were conducted at Maroochy Research Facility in Queensland in an environmental controlled glasshouse maintained at temperatures between 24°C and 30°C. The cultivars Kabarla (susceptible) and Festival (resistant) were used as the standards (Hutton and Gomez 2006).

Experiment 1: In June 2010, five strawberry cultivars; Albion, Camarosa, Festival, Fortuna and Kabarla, were inoculated with Qld *Fof* isolates N15309 and N13581. The cultivars Albion, Camarosa and Festival were selected as they are commonly grown in the major production areas of Australia, while 'Kabarla' has been significant in Qld. 'Albion' and 'Camarosa' were also of interest due to the high losses that occurred recently in California (Koike, 2009). 'Fortuna' was selected as it is becoming popular in both WA and Qld in commercial production. Disease development was monitored weekly and visual disease severity ratings taken from 4 weeks after inoculation. A completely randomised design with six plants (replicates) of each cultivar and six non-inoculated 'Kabarla' plants (controls) were used for each isolate treatment. Ratings for visual disease severity were taken at seven time points (weeks 4 to 10 post-inoculation).

Experiment 2: In October 2012, eight cultivars; Camarosa, Earliblush, Festival, Fortuna, Kabarla, Redlands Joy, Rubygem and Sugarbaby were inoculated with five isolates of *Fof* (three Qld isolates N15309, N13581, N18582 and one WA isolate N18462). The cultivar Camarosa was again included due to the severe losses suffered recently in WA from Fusarium wilt. A completely randomised design with nine plants (replicates) of each cultivar and six non-inoculated 'Kabarla' plants (controls) were used for each isolate treatment. Ratings for disease severity were taken at ten time points (weeks 3 to 12 post-inoculation).

Experiment 3: In May 2013, five cultivars cultivars; Camarosa, Festival, Fortuna Earliblush and Kabarla, were inoculated with four isolates of *Fof*. This experiment included two south Australian (SA) isolates; SA126 and SA127 along with N18462 (WA origin) and N13581 (Qld origin). The isolates SA126 and SA127 had been harvested from strawberry plants showing Fusarium wilt symptoms. Both isolates were identified by morphological characteristics as *F. oxysporum*; however, pathogenicity had not been previously tested. Because commercial runners of some cultivars were not available, cultivar choice was limited to one known susceptible and one resistant (Table 4.2). An incomplete block design with eight plants (replicates) of each cultivar and five non-

inoculated ‘Kabarla’ plants (controls) were used for each isolate treatment. Ratings for disease severity were taken at nine time points (weeks 2 to 10 post-inoculation).

Table 4.2 *Fof* isolates and strawberry cultivars used in Experiment 3.

Isolate	**Cultivars**				
SA126	Camarosa	Festival	Kabarla		
SA127		Festival	Kabarla		
N13581	Camarosa	Festival	Kabarla	Earliblush	Fortuna
N18462				Earliblush	Fortuna

4.2.3 Plant inoculation

Plants (≈ 6 months old) were removed from their pots, the crown and roots were washed free of soil and debris and in a randomised order immersed into the *Fof* spore suspension for 10 mins, after which they were potted into 100mm × 140mm pots using potting medium previously described (Figure 4.1a). Approximately 10mm of sterile gravel (3 to 5mm in diameter) was added around the plant on top of the soil mix to prevent splash (Figure. 4.1b). Control ‘Kabarla’ plants were similarly immersed in sterile water only. Plants were randomly allocated, spaced 25 to 30cm apart on a heated bench at 28°C in a glasshouse with natural daylight. The control plants (no *Fof* inoculum) were separated from inoculated plants by approximately 1m to avoid contamination. Plants were watered to free draining with tap water daily for the first 5 days, and three times per week thereafter, and fertilised at 2 week intervals with ‘Yates Aquasol Soluble’ fertiliser at the recommended rates.

(a)

(b)

Figure 4.1 (a) Kabarla' plants immersed in a container of *Fof* inoculum. (b) Post-inoculation, plants placed in pots using potting mix, with sterilised gravel on top.

4.2.4 Disease severity assessment

Disease development was monitored on the individual plants and visual severity ratings taken at weekly intervals post-inoculation. Severity of foliar symptoms was assessed on a 0 to 10 disease visual index (Hutton and Gomez, 2006) as described in Chapter 2.

The degree of resistance (*x*) to *Fof* was determined from the mean disease severity score by the following scale:

$x \leq 2$ = resistant
$2 < x \leq 4$ = moderately resistant
$4 < x \leq 6$ = moderately susceptible
$6 < x \leq 8$ = susceptible, $x > 8$ = very susceptible

4.2.5 Sampling

Symptomatic plants, representing all experiments, were sampled at 10 to 12 weeks post-inoculation. The crowns were washed clean and surface sterilised using sodium hypochlorite and rinsed three times in sterile water. Crowns were cut in cross section and discoloured pieces of crown plated onto 1/4 strength potato dextrose agar and incubation at 24°C.

4.2.6 Statistical analysis

A mean disease severity rating for each inoculation treatment was calculated across replicates. Data were analysed in GenStat (version 11.1) (VSN International Ltd) using ANOVA, on the estimate of Fisher's protected least significant difference test ($P<0.05$).

To further analyse cultivar × isolate interactions, mixed models with smoothing splines in ASReml-R package (Butler *et al.*, 2009) were fitted to look at the responses over time using data from experiment '2'. The times were treated as equally spaced from 1 to 10 and the intercepts were predicted at the midpoint of time (time 5).

4.3 Results

Experiment 1: Plants of the cultivar Kabarla from both isolate treatments were first to show symptoms of Fusarium wilt, exhibiting chlorosis and necrosis of the leaves, lesions on the petioles, and the wilting and collapse of petioles and leaves (Table 4.3). Results from repeated measures analysis across times show that there was a significant cultivar × time interaction, where the cultivars performed differently over time (Figure 4.2). At 10 weeks post-inoculation, most 'Kabarla' plants were either dead or severely wilted.

'Kabarla' and 'Albion' showed susceptibility to both *Fof* isolates, while Camarosa, Festival, and Fortuna, demonstrated resistance (Table 4.3). A second analysis (ANOVA at a single time point) performed on the data from the final rating time, 10 weeks, show there was no significant difference between isolates and no significant isolate by cultivar interaction, but that there was a significant cultivar effect (Tables 4.4 and 4.5).

Table 4.3 Assessment of strawberry cultivar responses to *Fof* isolates N15309 and N13581 (experiment 1), using means of disease severity ratings taken at 10 weeks post-inoculation.

Cultivar	**Isolate**	**No. plants exhibiting disease symptoms (n=6)**	**Disease rating (means)[a]**
Kabarla	N13581	6	8.8
Kabarla	N15309	6	10.0
Albion	N13581	4	5.3
Albion	N15309	5	6.8
Camarosa	N13581	1	0.3
Camarosa	N15309	2	3.0
Fortuna	N13581	2	1.5
Fortuna	N15309	1	0.7
Festival	N13581	0	0.2
Festival	N15309	2	1.8

[a]Treatment means of all plants inoculated with *Fof*. (0 = healthy plants, showing no symptoms and 10 = plant death). LSD cultivar = 2.15, LSD isolate = 1.36, LSD cultivar.Isolate = 3.04

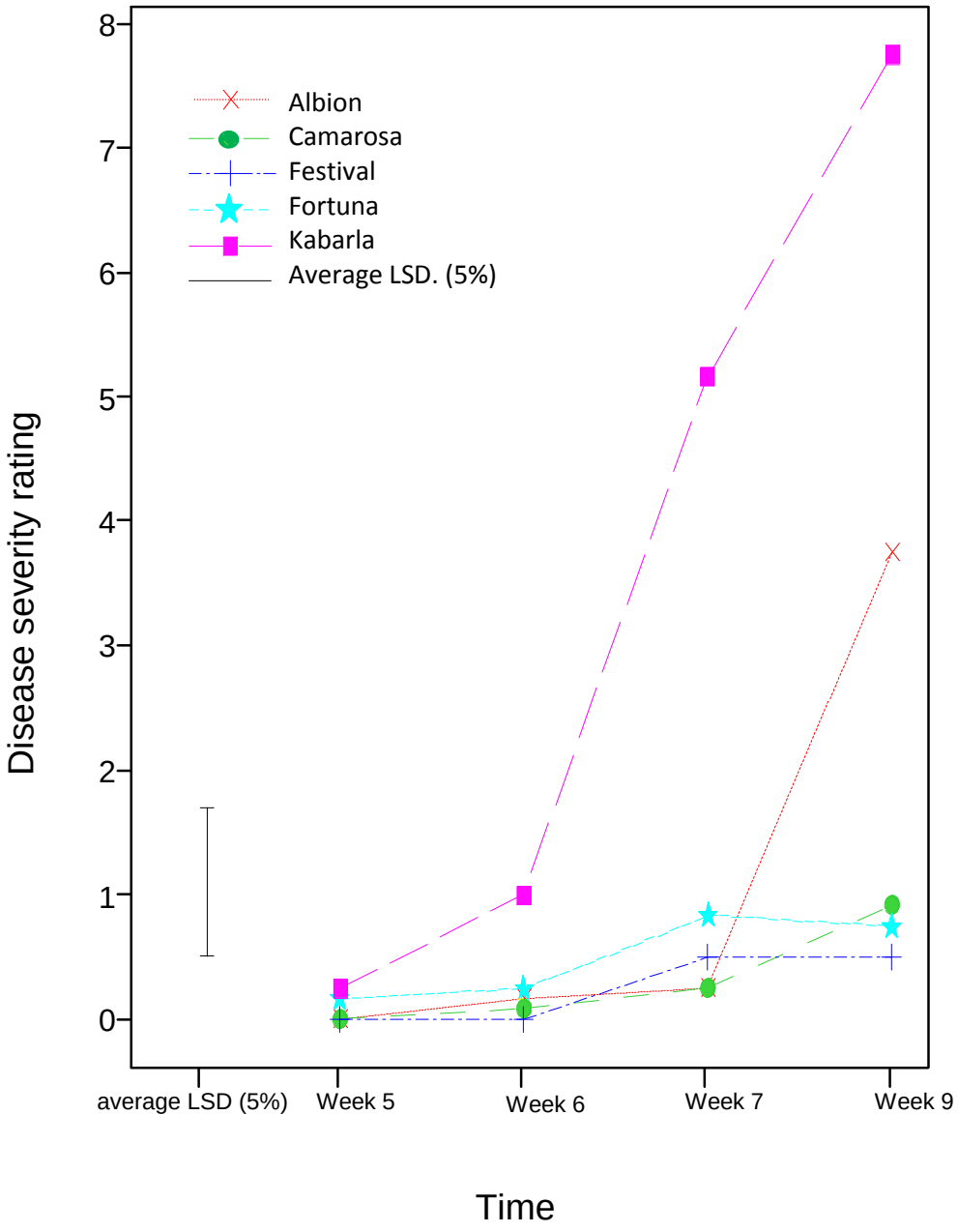


Figure 4.2 Means of disease severity rating taken from cultivars inoculated with N15309 and N13581 for time (week of rating) at different levels of cultivar. (F-test 31.75 on 8 and 48918 df, probability <0.001, epsilon 0.4793).

Table 4.4 Ranked means for disease severity ratings of two *Fof* isolates across five strawberry cultivars (experiment 1). LSD = 1.36.

Isolate	Ranked means
N15309	4.47
N13581	3.23

F-test is not significant at the P = 0.050 level

Table 4.5 Ranked means for disease severity ratings of five cultivars tested with *Fof* isolates (experiment 1). LSD = 2.15.

Cultivar	Ranked means
Kabarla	9.42[a]
Albion	6.08[b]
Camarosa	1.67[c]
Fortuna	1.08[c]
Festival	1.00[c]

Means with same superscript are not significantly different at the P = 0.05 level

Experiment 2: By 12 weeks post-inoculation all plants of 'Camarosa' × N18462, with the majority of plants of 'Fortuna', 'Earliblush' and 'Kabarla' × N18462, and plants of 'Kabarla' × N13581, showed strong symptoms of Fusarium wilt (Figure 4.3). Plants of 'Fortuna' showed moderate susceptibility over all isolates while 'Festival' and 'Sugarbaby' showed resistance (Table 4.6). Plant mortality of 'Festival' occurred in only one plant × the isolate N18582. Isolate N18462 resulted in higher disease severity ratings across most cultivars except 'Festival' and 'Rubygem' with zero ratings with this isolate (Figure 4.3). There were significant ($P < 0.05$) cultivar effects from 3 weeks post-inoculation, then significant ($P < 0.05$) cultivar × isolate interaction from 7 weeks post-inoculation.

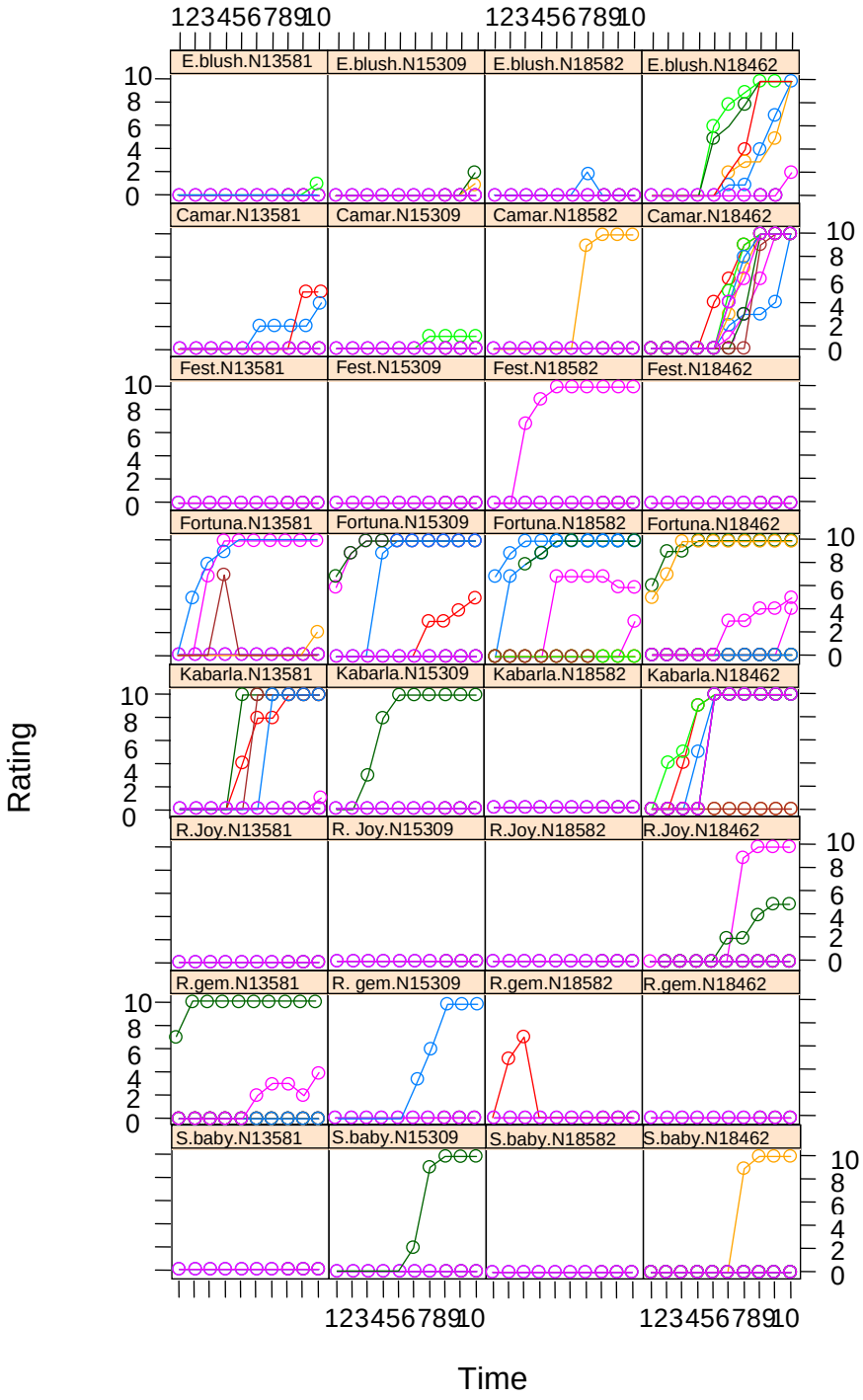


Figure 4.3 Means of disease severity for eight cultivars of strawberry inoculated with four isolates of *Fof*, taken at 10 time points (weeks 3 to 12). (Camar=Camarosa, E.blush=Earliblush, Fest=Festival, R.Joy=Redlands Joy, R.gem=Rubygem, S.baby-Sugarbaby).

Table 4.6 Means of visual rating for disease severity (where 0=healthy plant, and 10= plant death, section 2.2.3.1) from eight cultivars of strawberry inoculated with four *Fof* isolates, taken at 12 weeks post-inoculation**.**

Cultivar	Isolate			
	N13581	**N15309**	**N18582**	**N18462**
Camarosa	1.0[a]	0.1[a]	1.1[a]	10.0[d]
Earliblush	0.1[a]	0.4[a]	0.0 [a]	8.2[d]
Festival	0.0[a]	0.0[a]	1.1 [a]	0.0 [a]
Fortuna	2.4[abc]	3.9[bc]	4.3[c]	4.3[c]
Kabarla	4.6[c]	1.1[a]	0.0 [a]	7.8[d]
Redlands Joy	0.0[a]	0.0[a]	0.0 [a]	1.7[ab]
Rubygem	1.6[ab]	1.1[a]	0.0 [a]	0.0 [a]
Sugarbaby	0.0[a]	1.1[a]	0.0 [a]	1.1 [a]

Means with same superscript are not significantly different at the P = 0.05 level

Standard error of differences: cultivar = 0.6, isolate = 0.4, cultivar.isolate = 1.3

Experiment 3: At 10 weeks post-inoculation the majority of plants of 'Kabarla' inoculated with N13581 and SA126 were showing symptoms including some dead plants. Cultivars inoculated with isolate SA127 remained healthy with only one Kabarla plant showing very mild symptoms. Differences for isolate × cultivar were significant (P < 0.05) (Table 4.7).

Table 4.7 Means of visual rating for disease severity taken at 10 weeks post-inoculation from five cultivars of strawberry inoculated with four *Fof* isolates.

Cultivar	Isolate			
	SA126	**SA127**	**N13581**	**N18462**
Camarosa	0.6[cd]	N/T	2.5[cd]	N/T
Earliblush	N/T	N/T	3.1[bc]	0.5[cd]
Festival	0.0[d]	0.0[d]	1.3[cd]	N/T
Fortuna	N/T	N/T	3.0[c]	1.3[cd]
Kabarla	5.8[ab]	0.8[cd]	7.9[a]	N/T

Means with same superscript are not significantly different at the P = 0.05 level

Standard error of differences: cultivar = 1.0, isolate = 0.9 cultivar.isolate =1.4

N/T = not tested

4.3.1 Summary of experiments

Cultivar responses across the three experiments were determined based on the disease severity assessment (section 4.2.4) (Table 4.8).

Table 4.8 Responses of nine cultivars across the three experiments for strawberry cultivars inoculated with *Fof* isolates.

Cultivar	N13581 N15309	N18582	N18462	SA126
Albion	Susceptible	N/T	N/T	N/T
Camarosa	Resistant	Resistant	Very Susceptible	Resistant
Earliblush	Resistant	Resistant	Very Susceptible	N/T
Festival	Resistant	Resistant	Resistant	Resistant
Fortuna	Resistant	Mod.resistant	Mod.resistant	N/T
Kabarla	Very Susceptible	Resistant	Susceptible	Mod.susceptible
Redlands joy	Resistant	Resistant	Resistant	N/T
Rubygem	Resistant	Resistant	Resistant	N/T
Sugarbaby	Resistant	Resistant	Resistant	N/T

N/T = not tested

4.3.2 Sampling

In the three experiments, *Fof* was re-isolated from crowns of symptomatic plants. The severity of discolouration in the vascular tissues of the crown was consistent with that of foliar disease severity. Typically plants showing no symptoms had clean, disease free crowns, while those showing symptoms exhibited vascular discolouration and rots. From crown isolations, significant fungal colonies, identified by morphological characteristics as *F. oxysporum,* confirmed effective inoculation. All the control plants were healthy and free of symptoms, with no internal discolouration.

4.3.3 Cultivar × Isolate interactions

There was a significant interaction between the cultivar and the isolates (i.e., the cultivars performed differently in their disease response depending on what isolate was used) and this was evident at the intercept level (the mean level of disease half way through the experiment, i.e., at time 5), slope level (how fast the disease is increasing over time), and also the actual shape of the disease profile over time (Figure 4.1). The shape of some of the cultivar × isolate disease profiles were flat across time (zero disease rating) while

others increased in disease over time and tapered off to an asymptote, or just continued increasing (Appendix 4.2).

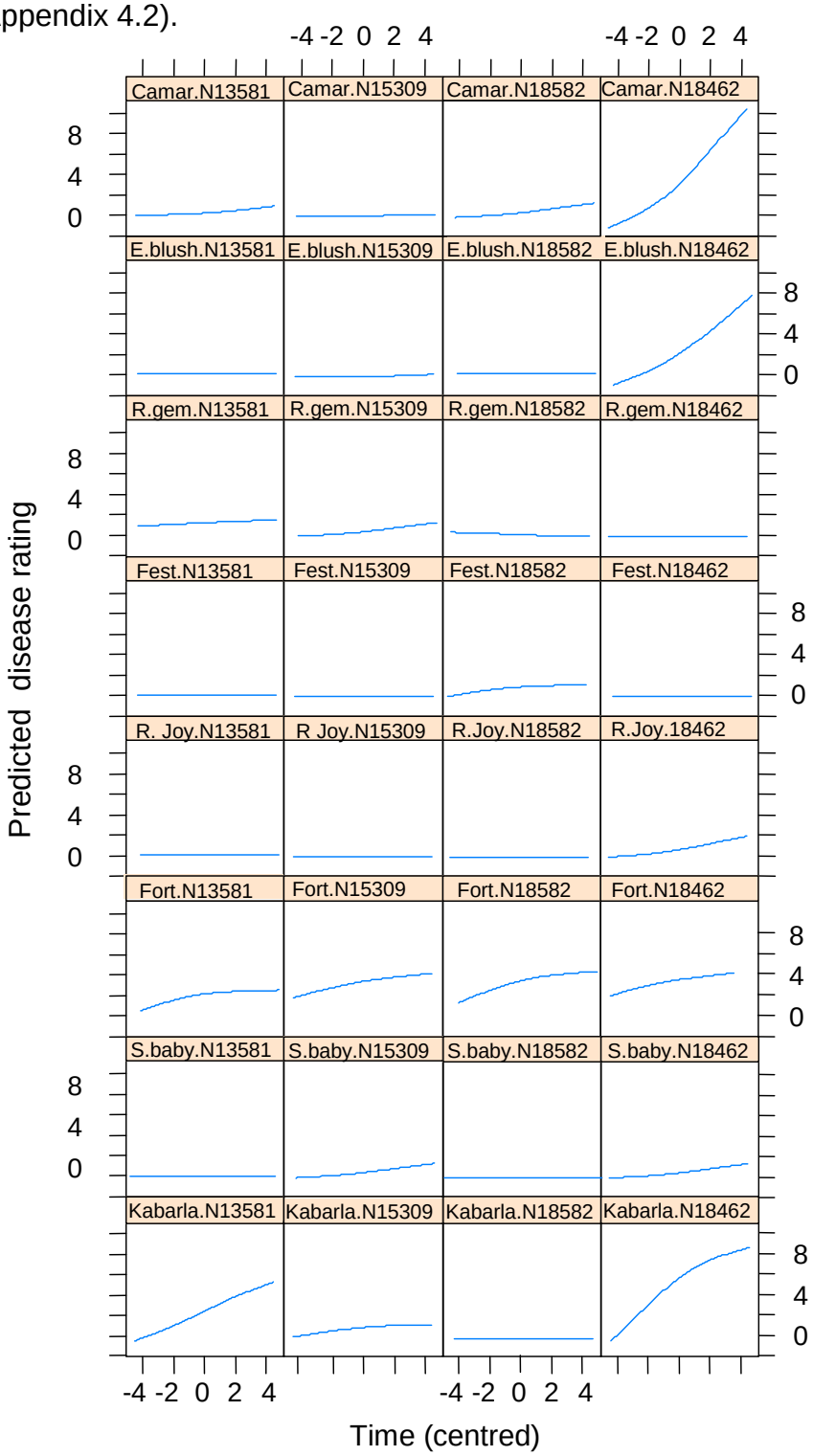


Figure 4.4 Analysis across times for cultivar × isolate response to *Fof* (experiment 2) using smoothing splines in ASReml. (Camar=Camarosa, E.blush=Earliblush, Fest=Festival, Fort= Fortuna, R. Joy=Redlands Joy, R.gem=Rubygem, S.baby=Sugarbaby).

4.4 Discussion

For the assessment of strawberry cultivars with different isolates of *Fof* using a disease severity rating of 1 to 10 in a controlled glasshouse environment, the mean disease ratings of the cultivars inoculated varied. Notably in experiment 2, where there was a significant variation in the virulence of the isolates tested, significant cultivar differences, significant cultivar × time differences, and significant cultivar differences across isolates ($P \leq 0.05$), indicating that a cultivar × isolate interaction may exist. Race structure or cultivar specificity exists for most *formae speciales* of *F. oxysporum* (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; Fourie *et al.*, 2011). For example, race structure exists for *F. oxysporum* f. sp. *lycopersici* in tomato, *F. oxysporum* f. sp. *melonis* in melon and *F. oxysporum* f. sp. *cubense* in banana. Therefore, race structure for *Fof* is very plausible. All isolates showed some degree of pathogenicity to strawberry cultivars tested except isolate SA127 (SA origin), which was therefore considered non-pathogenic. All 'Kabarla' plants inoculated with *Fof* isolates N13581, N15309 (Qld origin), N18462 (WA origin) and SA126 (SA origin) showed strong symptoms of Fusarium wilt, demonstrating a high level of susceptibility. Similarly, most inoculated 'Festival', 'Sugarbaby' and 'Rubygem' plants were healthy, showing a high degree of resistance, with a few only showing mild symptoms. These results are consistent with those of Hutton and Gomez (2006).

The differences in mean ratings among the two isolates tested in experiment 1 (N13581 and N15309) are not significantly different. Both isolates were of Qld origin and belong to the same VCG (A) (Chapter 2, Table 2.1) and are therefore genetically similar. Assessments of cultivar response for both isolates in experiment 1 are shown in Table 4.5. Where these two isolates were tested in experiment 2 against the isolate N18462, which belongs to a separate VCG (B) (Chapter 2, Table 2.1), the disease mean ratings are significantly different (Table 4.6). For example, in experiment 1 the cultivar Camarosa inoculated with isolates N13581 and N15309 (of Qld origin and belonging to VCG A) had ratings of 0.3 and 3.0 respectively (resistant) while in experiment 2, Camarosa inoculated with N18462 (of WA origin and belonging to VCG B) had a mean disease rating of 10.0 (susceptible).

In experiment 1, the cultivar Fortuna was rated as resistant, having mean ratings below 2 (resistant) for both isolates tested, whereas plants of Fortuna in experiments 2 and 3 had mean ratings between 2 and 4 (moderately resistant) to the same isolates tested. Similarly, differences in ratings for the cultivar Kabarla existed between experiments 1

and 2. In experiment 1 the mean rating for Kabarla by both isolates was greater than 8 (very susceptible), while in experiment 2, Kabarla × N13581 has a mean rating of 4.6 (moderately susceptible) and Kabarla × N15309 a mean rating of only 1.1 (resistant). These differences may have been due to escapees and the unpredictability of the pathogen to mutate (Burger *et al*., 2003; Fourie *et al*., 2011), as some plants were dead (rating 10) while others where healthy (rating 0). However, typically the cultivar Kabarla was susceptible across experiments. 'Albion' was tested in experiment 1 only and with only two isolates in 2010, further testing on a wider range of isolates would be necessary in determining this cultivars resistance status.

The Western Australian *Fof* strain N18462 resulted in higher ratings (susceptible) across most cultivars however Festival and Rubygem had low ratings (resistant) with this isolate. The isolate N18462 was the most aggressive isolate of the samples that were tested. Additionally, it seems particularly well adapted to attack 'Camarosa', which is very susceptible and the most prominent strawberry planted in WA. In WA, typically a mono-cultural system is practised using the same soil year after year and no cover cropping practiced. With the likelihood of infected plant material carrying over from season to season, WA could be at risk of increased outbreaks of Fusarium wilt.

Fitting smoothing splines (in ASReml) made up of an intercept, a linear slope component, and a curvature (spline) to model the cultivar × isolate combination disease response provides an idea of what is happening over time. Looking at the different disease profiles over time for each cultivar × isolate (Figure 4.3), not all plots of one cultivar (for the four different isolates) behave the same and clearly some isolates affect some cultivars differently than others. The analyses show a significant cultivar × isolate interaction for intercepts, slopes, and curvature of the splines (Figure 4.3). For example, 'Kabarla' had low disease with isolate N18582 but had high disease with isolate N18462. The linear slope component indicates how quickly the level is increasing or decreasing around the mean level. The curvature (spline), or the smooth non-linear sections, show a number of the responses are not linear (not a straight line over time) and follow a curve over time. The intercepts are predicted at the midpoint of time (at rating time 5), so reflect the predicted level of disease midway through the experiment. For assessment of the most resistant cultivars, a low intercept is best and many of these intercepts are actually zero (all zero ratings).

In conclusion, there is clearly variation in cultivar susceptibility to the isolates used in this study. In addition, a cultivar × isolate interaction is happening within the dynamics of susceptibility or resistance of cultivars to different strains of *Fof*. For breeding new elite cultivars with increased *Fof* resistance, the cultivars Festival, Sugarbaby, and Rubygem would be a first choice as suitable parents, and testing with a number of isolates is recommended. From the results of the cultivar response experiments, the cultivars Festival and Sugarbaby (resistant), and Kabarla (susceptible) were chosen to be used in crossings to generate progeny for the screening described in chapter 5.

The existence of a potential race structure, pathogenic variability and the mutability of the pathogen is a basis for continued research, using a larger sample of isolates and cultivars across environments. This would be beneficial for the development of durable resistant cultivars.

5.0 Chapter 5 Manuscript: Resistance to *Fusarium oxysporum* f. sp. *fragariae* and predicted breeding values in strawberry

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Resistance to *Fusarium oxysporum* f. sp. *fragariae* and Predicted Breeding Values in Strawberry

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ADDITIONAL INDEX WORDS. BLUP, *Fragaria* × *ananassa*, fusarium wilt, heritability, linear mixed model, susceptible

ABSTRACT. Fusarium wilt of strawberry, incited by *Fusarium oxysporum* f. sp. *fragariae* (*Fof*), is a major disease of the cultivated strawberry *(Fragaria* ×·*ananassa*) worldwide. An increase in disease outbreaks of the pathogen in Western Australia and Queensland plus the search for alternative disease management strategies place emphasis on the development of resistant cultivars. In response, a partial incomplete diallel cross involving four parents was performed for use in glasshouse resistance screenings. The resulting progeny were evaluated for their susceptibility to *Fof*. Best-performing progeny and suitability of progenies as parents were determined using data from disease severity ratings and analyzed using a linear mixed model incorporating a pedigree to produce best linear unbiased predictions of breeding values. Variation in disease response, ranging from highly susceptible to resistant, indicates a quantitative effect. The estimate of the narrow-sense heritability was 0.49 ± 0.04 (SE), suggesting the population should be responsive to phenotypic recurrent selection. Several progeny genotypes have predicted breeding values higher than any of the parents. Knowledge of *Fof* resistance derived from this study can help select best parents for future crosses for the development of new strawberry cultivars with *Fof* resistance.

Fusarium vascular wilt disease is incited by pathogens of *Fusarium oxysporum* and comprises major soil-borne fungal pathogens of many important crops. The *F. oxysporum* complex comprises both pathogenic and non-pathogenic strains, many being indistinguishable using phenotypic characters. Pathogenic strains are grouped into formae speciales depending on host specificity. *F. oxysporum* f. sp. *fragariae* is specific (pathogenic) to strawberry and considered a worldwide major disease of the cultivated strawberry. *Fof* was first identified and described in 1962, when it spread rapidly in the strawberry production areas of Brisbane, Queensland, Australia (Winks and Williams, 1965). Subsequently, *Fof* has been reported in many countries including Japan (Mori et al., 2005; Takahashi et al., 2003), Mexico (Da´valos-Gonza´lez et al., 2006), Korea (Nagarajan et al., 2006), China (Zhao et al., 2009), Spain (Arroyo et al., 2009), and the United States (Koike, 2009).

Fusarium oxysporum species reproduce asexually, producing microconidia, macroconidia, and chlamydospores. Chlamydospores can remain viable in the soil for many years (Smith and Snyder, 1975) making fusarium wilt diseases difficult to control. Pathogenic strains enter the host through the roots moving into the vascular system where it colonizes in the xylem vessels and impedes water movement, causing the plant to wilt and die (Lagopodi et al., 2002; Xiao-min et al., 2011). Newly infected plants may die within a few weeks if weather conditions are hot and wet (Hancock, 1999; Winks and Williams, 1965). Large areas of strawberry production can rapidly succumb to this disease.

Commercial strawberry growers have typically relied on disease-free runners and the pre-plant soil fumigant methyl bromide as their major management strategies for soilborne pathogens, including *Fof*. Outbreaks of fusarium wilt were relatively uncommon under a regime of methyl bromide fumigation so that neither the disease nor resistance breeding was considered important. However, after the phase-out of methyl bromide in 2005 under the ‘‘Montreal Protocol on Substances That Deplete the Ozone Layer,’’ outbreaks of the disease have caused up to 50% plant mortality in some fields in the Perth district of Western Australia (Golzar et al., 2007) and up to 10% mortality in the southeastern regions of Queensland. The incidence of severe outbreaks is likely to increase in subtropical Queensland if susceptible cultivars become popular for marketing reasons. Currently, there is no effective treatment for *Fof*-infected plants, and cultivars with resistance to fusarium wilt are required to limit disease outbreaks (Da´valos-Gonza´lez et al., 2006; Herrington et al., 2007).

As a result of the lack of effective fumigants and also the large plant losses occurring from fusarium wilt in Australia, the Queensland strawberry breeding program identified a need to include fusarium wilt resistance as part of the primary selection criteria of its multi-trait breeding strategy. Varietal field screenings for susceptibility to fusarium wilt began in 2002 at Maroochy Research Facility, Nambour, Queensland. Screening of cultivars in Fof-infested field plots identified large variation in cultivar response. Assessed on a 0 to 10 symptom severity scale (Hutton and Gomez, 2006), the cultivars Maroochy Jewel and Kabarla were described as susceptible (greater than 5) and ‘Festival’ and ‘Sugarbaby’ as resistant (1 or less) (Hutton and Gomez, 2006). Further glasshouse experiments, testing nine cultivars for symptoms of *Fof*, confirmed ‘Kabarla’ and ‘Camarosa’ as susceptible to fusarium wilt and ‘Sugarbaby’ and ‘Festival’ as resistant (M.L. Paynter, unpublished data). Additionally, a study conducted recently in Western Australia

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also described 'Festival' as resistant to fusarium wilt and 'Camarosa' as susceptible (Fang et al., 2012). 'Camarosa' (susceptible) is the major cultivar grown in Western Australia, and its high susceptibility may partially explain the greater losses to fusarium wilt experienced in Western Australia.

The genetic base in the cultivated strawberry is relatively narrow (Sjulin and Dale, 1987); however, high levels of heterozygosity in the strawberry genome and the hybrid nature of *F.* ×*·ananassa* (Hancock, 1999; Maas, 1998) make breeding for disease resistance a viable option. Despite much knowledge on fusarium wilt diseases in other crops, little is known of the genetics of resistance in strawberry, where most inheritance studies on strawberry populations have been on fruit traits (Murti et al., 2012; Shaw and Sacks, 1995; Verma et al., 2003). From a study in Japan of *Fof* resistance in strawberry, Mori et al. (2005) reported bimodal segregation of disease resistance to fusarium wilt in F_1 hybrid seedlings of strawberry and concluded that major genes were involved, but there was also a multigenic component, because among susceptible cultivars, the disease severity index varied continuously. Continuous variation in resistance to *Fof* among strawberry cultivars has also been observed by Hutton and Gomez (2006). Information about the heritability of the resistance in strawberry and estimation of the breeding value of individual plants would be beneficial in identifying highly resistant genotypes and using them in breeding programs.

Best linear unbiased predictions [BLUPs (Henderson, 1984)] of breeding values have been used in many breeding programs to increase the frequency of desired phenotypes in progeny (Davik and Honne, 2005; Hardner et al., 2012; Kennedy, 1981). Use of specialized crossing designs (e.g., full or partial diallel) and statistical models can generate individual breeding values, and so determine their suitability as parents, from an observed sample of progeny. If pedigree data are included in the model, the information on the relatedness of the genotypes allows better estimates of total genetic effects and predicted breeding values because the effective number of observations available increases (White and Hodge, 1989). Fitting models with pedigree information to estimate genetic effects have been used for apple [Malus ·domestica (Durel et al., 1998)], peach [Prunus persica (de Souza et al., 1998)], and strawberry (Davik and Honne, 2005). Strawberry is an ideal crop for selection using predicted breeding values because the short generation interval allows for progeny measurements to be performed and available for analysis within one season.

In this report, we used progeny resulting from a partial incomplete diallel crossing to test for resistance to fusarium wilt to obtain estimates of individual predicted breeding values and genetic parameters relevant to fusarium wilt resistance in our strawberry population. This knowledge can be used to enable better predictions about progeny response to selection for the resistance trait and assist in the breeding of strawberry cultivars with increased resistance to fusarium wilt.

Materials and Methods

Genetic material

Four parents were hand-pollinated in 2009 using a partial incomplete diallel cross design to generate 245 progeny from 14 full-sib families, numbering from six to 27 progeny per family (Table 1). The parents chosen for crossing were derived from ancestors containing several connecting relatives (pedigree linkages) and were considered representative of a diverse range of Fof susceptibility. In Feb. 2011, up to five freshly emerged runners from each progeny were potted into 100 · 140-mm plastic pots (one plant per pot) containing steam-sterilized potting mix composed of double-washed river sand and coir (by volume:1:1) with a pre-mixed fertilizer of (in g_L–1) 5.1 nitrogen, 7.2 phosphorus, 4.6 potassium, 60.4 calcium, 0.08 copper, 0.06 iron, 0.32 magnesium, and 0.15 zinc.

Table 1. Crossing scheme used in screening for resistance to *Fusarium oxysporum* f. sp. *fragariae*.[z]

Family	Parent cultivars used in cross		Progeny (no./family)
	Female	Male	
2772	Festival	Festival	15
2773	Festival	Maroochy Jewel	26
2774	Festival	Kabarla	22
2775	Festival	Sugarbaby	27
2776	Maroochy Jewel	Maroochy Jewel	19
2777	Maroochy Jewel	Festival	10
2778	Maroochy Jewel	Kabarla	14
2779	Maroochy Jewel	Sugarbaby	23
2780	Kabarla	Kabarla	22
2781	Kabarla	Festival	19
2782	Kabarla	Maroochy Jewel	14
2783	Kabarla	Sugarbaby	22
2786	Sugarbaby	Sugarbaby	6
2787	Sugarbaby	Maroochy Jewel	6

[z]Indicated are family number, parents used, and number of strawberry progeny per family.

The cultivar Kabarla, reported to be susceptible in the southeastern regions of Queensland (Hutton and Gomez, 2006), was used to validate the inoculation procedure. Certified disease-free 'Kabarla' runners were grown under the same conditions as described for strawberry progenies.

Fungal isolates and inoculum preparation

Two isolates of *Fof*, originally collected in Queensland, from the crowns of infected strawberry plants showing typical severe symptoms of fusarium wilt disease (N13581 harvested from 'Kabarla' in 2002 and N15309 harvested from 'Camarosa' in 2005), were used as inoculum in screening trials. These were obtained from the Maroochy Research Facility culture collection at Nambour. Both isolates belonged to the same vegetative compatibility group and had been identified as *Fof* based on spore and colony morphology, cultural characteristics, and their high virulence confirmed by pathogenicity tests performed at the Maroochy Research Facility (M.L. Paynter, unpublished data). Cultivar responses of these two highly pathogenic isolates of *Fof* were confirmed by previous trials (M.L. Paynter, unpublished data) and results were similar to Fang et al. (2012) and Hutton and Gomez (2006).

For preparation of inocula, single spore accessions of N13581 and N15309 were plated onto one-quarter strength potato dextrose agar amended with 50 mg_L^{-1} streptomycin sulphate and incubated at 24 ºC for 2 weeks. The spores were collected from culture plates after addition of sterile deionized water and rubbing the agar surface with a glass spreader. The spore suspension was then filtered through four layers of cheesecloth. The conidial concentration (mainly microconidia and macroconidia with some chlamydospores) was determined using a hemocytometer and adjusted with sterile water to 1×10^6

conidia/L. Before use, both N13581 and N15309 conidial suspensions were combined equally.

The inoculation procedure combined a root dip technique (Pastor-Corrales and Abawi (1987) with the addition of sterilized ryegrass seed (Lolium perenne cv. Tetila) to assist the pathogens survival and proliferation (Smith et al., 2008). To prepare sterilized ryegrass seed, the ryegrass seed was rinsed under running tap water, drained, and soaked overnight in distilled water, after which the water was strained off and the seed rinsed several times with tap water until water was clear. The seed was then autoclaved at 121 ºC for 20 min on 3 consecutive days.

Inoculation process

In Nov. (late spring) 2011, plants were removed from their pots and 3 cm cut from the bottom of the root ball (soil and roots). The 3-cm section was put back into the pot and 20 mL of sterilized ryegrass seed was spread on top. The plants were inoculated in a randomized order by immersing the plant root ball in the inoculum (to 1 cm past the top of crown) for 10 min and then placed back (on top of ryegrass) into their pot. The plants were then firmly situated in their pots using potting medium (as described previously). Approximately 1 cm of sterile gravel (3 to 5 mm in diameter) was added around the plant on top of the soil mix to prevent splash. To validate the inoculation procedure, commercial runners of 'Kabarla' were used as positive and negative controls. Six 'Kabarla' were inoculated as described previously, whereas another six plants were immersed in sterile water only. Additionally, one to two plants from each family were also inoculated using sterile water only. Plants were randomly allocated, spaced at 25 cm apart, onto five heated benches at 30 ºC in a glasshouse. Non-inoculated control plants were placed on a bench separated from inoculated plants by 1 m to avoid contamination from splash and insects. Plants were watered with tap water daily for up to 5 d and approximately three times per week thereafter and fertilized at 2-week intervals. The trial design was an incomplete block design. There were between two to five replicate plants (ramets) per progeny with a mean number of four.

Disease severity assessment

VISUAL ASSESSMENT. Disease development was monitored weekly on the individual plants and visual severity ratings taken from 4 weeks post-inoculation. Severity of foliar symptoms was assessed on a 0 to 10 disease severity score (Hutton and Gomez, 2006) where: 0 = plant healthy with erect growth and full vigor; 1 = plant healthy, with a smaller canopy and moderate vigor; 3 = plant with a slight wilt, with the lower leaves affected; 5 = plant with a moderate wilt, with the mature leaves collapsing but young leaves still healthy; 7 = plant with a severe wilt, with most of the plant collapsed and mature leaves desiccated; 9 = plant with a very severe wilt, with the entire plant collapsed and most of plant desiccated; and 10 = dead plant. A mean disease severity score for each progeny was calculated across replicates.

Classification of resistance. The degree of resistance (x) to *Fof* was determined from the mean disease severity score by the following scale: $x \leq 2$ = resistant, $2 < x \leq 4$ = moderately resistant, $4 < x \leq 6$ = moderately susceptible, $6 < x \leq 8$ = susceptible, $x > 8$ = very susceptible.

SAMPLING. Random sampling from symptomatic (six to nine) and healthy (one to two) plants of each family was performed at 14 weeks post-inoculation. The crowns were washed clean and surface-sterilized using sodium hypochlorite and rinsed three times in sterile water. Crowns were cut in cross-sections and discolored pieces of crown plated onto potato dextrose agar and incubated at 24 LC. After 1 week, plates were inspected and analyzed microscopically for the presence of *Fof.*

Statistical analysis

Analysis of the disease response from severity ratings taken at 8, 10, and 14 weeks post-inoculation was performed using a linear mixed model incorporating a pedigree (interline relationships) on individual plant records (Hardner et al., 2012; Henderson, 1975; Oakey et al., 2006). The pedigree information included ancestors traced back up to four generations. The model included terms for the random additive genetic effects for each of the genotypes (including parents), random family effects, residual non-additive genetic variance, replicate clone effects, and table (location) effects. Control data were not included in analysis as a result of their placement on a separate table to restrict contamination. The analyses were performed using the ASReml-R package (Butler et al., 2009), which provides residual maximum likelihood (REML) estimates of the variance components and BLUPs of the random effects in the mixed model.

To investigate the genetic effects influencing resistance to fusarium wilt, the disease severity scores for the screenings were analyzed using the following linear mixed model: $y = X\tau + Z_g u_g + Z o u_o + e$, where y is the vector of observed disease ratings, τ is a vector of fixed effects (e.g., overall mean) with design matrix X, u_g is a vector of random total genetic effects with design matrix Z_g, u_o is a vector of other non-genetic random effects (e.g., replicate and table effects) with design matrix Z_o, and e is the vector of random residual effects.

All random effects in the model are assumed to be normally distributed with mean zero and the three random effect vectors (u_g, u_o, e) are assumed pairwise-independent. The variance models for the random non-genetic and residual effects are given by: $Var(u_o) = \sigma_o^2 I \qquad Var(e) = \sigma_e^2 I$

The vector of total genetic effects can be partitioned into three components, namely additive, non-additive, and family genetic effects. That is: $u_g = u_a + u_{na} + Z_f u_f$, where u_a represents the vector of additive genetic effects with distribution $u_a \sim N(0, \sigma^2_a A)$ where A is the known additive relationship matrix based on the pedigree information, u_{na} represents the non-additive or residual genetic effects with distribution $u_{na} \sim N(0, \sigma^2_{na} I)$ and u_f represents the family genetic effects with distribution $u_f \sim N(0, \sigma^2_f I)$.

Using the REML estimates of the variance components in the linear mixed model, the narrow-sense heritability (proportion of additive genetic variance over the total variance) was estimated by: $h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2 + \sigma_e^2)$, where σ_a^2 is the additive genetic variance of the individual geno-types, σ_{na}^2 is the non-additive genetic variance, σ_f^2 is the genetic variance between families, and σ_e^2 is the variance of the random residuals. Breeding values were predicted for each of the 245 progeny and four parents obtained by the BLUPs. Broad-sense heritability was estimated as:

$$H^2 = (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2) / (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2 + \sigma_e^2)$$

Results

PROGENY RESPONSE. Four weeks post-inoculation, external foliar symptoms of fusarium wilt were apparent. These included wilting, stunting of leaves, and lesions on petioles. Typically, susceptible plants were showing symptoms by Week 6. Plant deaths of up to 13% were observed by Week 8 and 56% by Week 14. Disease severity ranged from plants showing no symptoms of wilting to the complete collapse and death of plants. Non-inoculated control plants showed no symptoms. The response to *Fof* in the population was quantitative with continuous variation in susceptibility ranging from mildly resistant to very susceptible. Families that scored a low mean visual rating (i.e., showed good resistance) included: 2772, 2775, and 2786. Those with high scores (i.e., highly susceptible) included: 2776, 2778, and 2782. The remaining families showed variation in disease expression to Fof from moderately resistant to susceptible as shown in Figure 1.

The crown isolations carried out on symptomatic plants produced significant fungal colonies that were identified by morphological characteristics as *F. oxysporum*. The severity of discoloration in the vascular tissues of the crown was consistent with that of foliar disease severity and typically plants showing no symptoms had clean, disease-free crowns, whereas those showing symptoms exhibited vascular discoloration and rots.

The BLUP estimates of breeding values at Week 14 post-inoculation for the four parental genotypes ranged from 2 to 9.6 as shown in Figure 2. Higher breeding value estimates signify individuals that will pass on greater susceptibility to the next generation. Low BLUP values indicate cultivars that will pass on good resistance. 'Maroochy Jewel' (9.61) and 'Kabarla' (7.85) were the most susceptible parental genotypes, whereas 'Festival' (2.04) and 'Sugarbaby' (2.42) were the most resistant.

The family mean breeding values predicted for disease severity for each of the 14 families covered a broad range from 1.52 to 9.43 (average SE of difference equal to 0.55) as shown in Figure 3. Several progeny from the families 2775, 2786, and 2772 had high predicted levels of resistance (i.e., low disease severity score), whereas progeny from the families 2780, 2778, 2782, and 2776 had the highest predicted levels of susceptibility (i.e., high disease severity score).

The range of BLUP breeding values across the 245 individual genotypes was from 0.62 to 10.15 (average SE of difference equal to 2.00). Among the most resistant 10% of progeny, the five best genotypes for resistance included: 2772-14, 2772-15, 2772-05, 2786-01 and 2775-30 as shown in Figure 4. The best predicted breeding value for the individual progeny for the resistance trait belonged to 2772-14, which had a breeding value of 0.62 as shown in Figure 4.

Narrow-sense heritability was estimated at 0.49 ± 0.04 (SE) for the severity of disease, indicating that the observed phenotypic variation was moderately influenced by genetic factors. Broad-sense heritability was estimated at 0.50 ± 0.04. Variance components estimated from the linear mixed model at 14 weeks post-inoculation included the additive, non-additive, family, replicate, residual, and table (glasshouse location) effects (Table 2). There is a high correlation of breeding values among the assessment dates 8, 10, and 14 weeks after inoculation as shown in Figure 5.

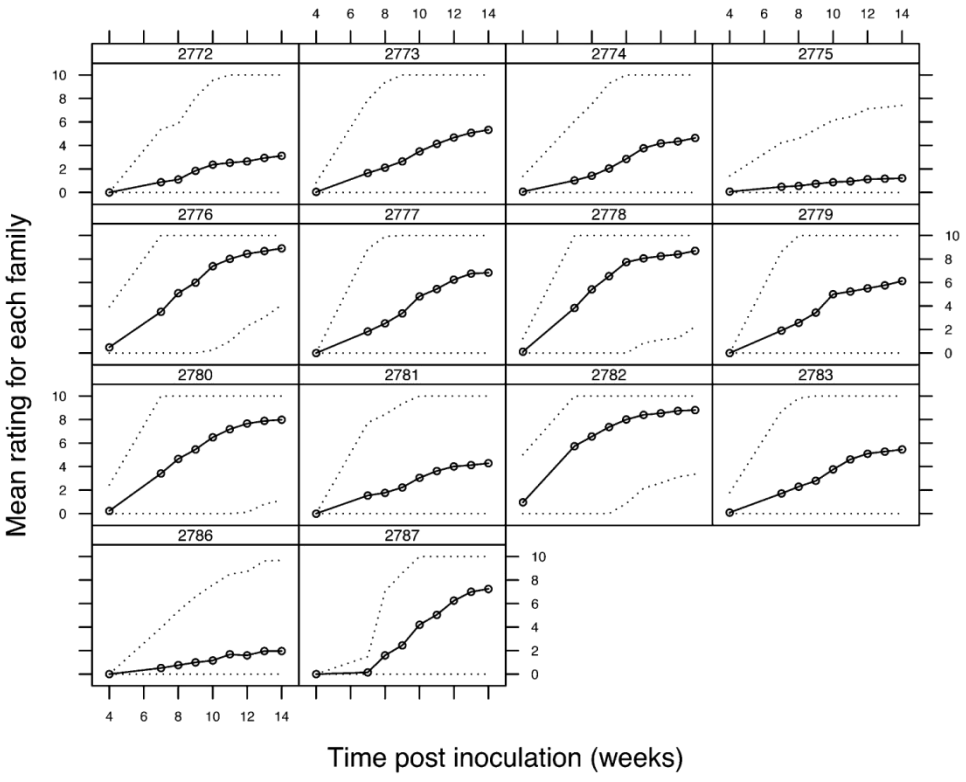

Fig. 1. Disease ratings for severity of disease symptoms in strawberry caused by *Fusarium oxysporum* f. sp. *fragariae* for each family taken at Weeks 4, 7, 8, 9, 10, 11, 12, 13, and 14 post-inoculation (0 = healthy plant and 10 = dead plant) showing variation among families over time. Each line represents the mean disease severity over time for each family. Dotted lines represent ± 2 SD from the mean.

The realized response to selection for selecting the best p^{th} percent (p%) of lines as parents can be calculated as the mean of the BLUPs of breeding values of the top p% of ranked cultivars (Cullis et al., 2006). Therefore, the realized response to selection for *Fof* disease rating by selecting the best (most resistant; i.e., the lowest disease ratings) 10% of progeny as parents (and assuming they are randomly mated) is given by 0.98. The overall population mean genetic effect for *Fof* rating before selection is 5.74. Hence, the predicted genetic gain in the first generation of selection is a decrease in disease rating of 4.76 or 83% of the original mean.

Discussion

This study aimed to deliver two fundamental objectives relevant to the breeding of fusarium wilt resistance in strawberry. The first was to evaluate strawberry progeny for their susceptibility to *Fof* and identify suitable parents for transferring the resistance trait. The second was to obtain estimates of genetic parameters (breeding values, variances,

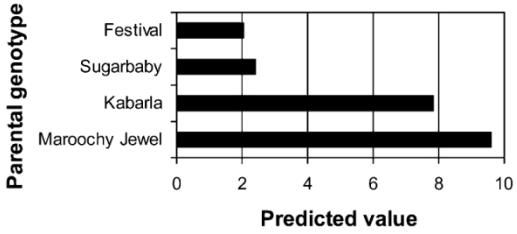

Fig. 2. Best linear unbiased prediction (BLUP) estimates of average breeding value for severity of disease symptoms in strawberry caused by *Fusarium oxysporum* f. sp. *fragariae* for parents taken at Week 14 post-inoculation. Parent BLUP average SE of differences = 0.78.

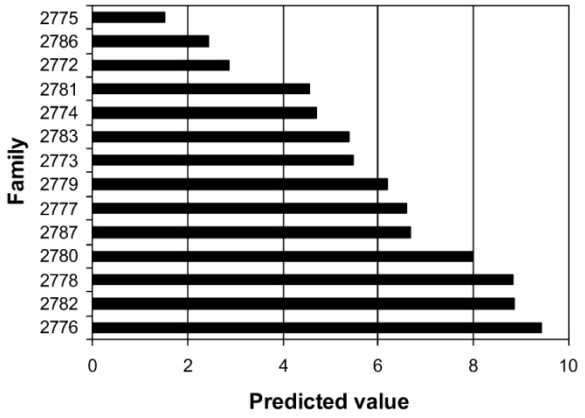

Fig. 3. Best linear unbiased prediction (BLUP) estimates of average breeding value for severity of disease symptoms in strawberry caused by *Fusarium oxysporum* f. sp. *fragariae* for each family taken at Week 14 post-inoculation. Family BLUP average SE of differences = 0.55.

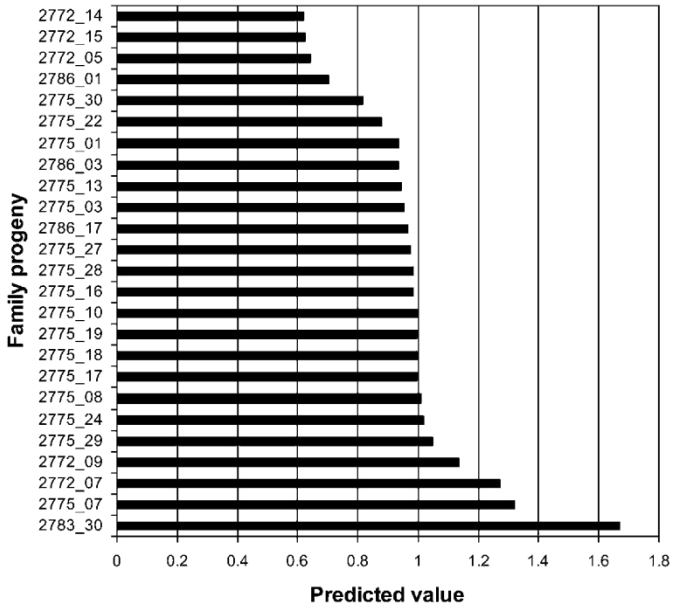

Fig. 4. Predicted breeding values (best linear unbiased predictions) of the most resistant (lowest symptom expression) 10% of strawberry progeny to *Fusarium oxysporum* f. sp. *fragariae* at 14 weeks.

Table 2. Table of residual maximum likelihood estimates of variance components (additive, non-additive, family, replicate, residual, and table effects) and SEs of strawberry progeny estimated from the linear mixed model fitted to *Fusarium oxysporum* f. sp. *fragariae* disease severity rating at 14 weeks post-inoculation.

Variance component			SE
Additive genetic variance	s_a^2	10.09	1.45
Non-additive genetic variance	s_{na}^2	0.00[z]	—
Family	s_f^2	0.08	0.24
Replicate (clone)		0.40	0.32
Bench (location in glasshouse)		0.02	0.07
Residual variance	s_e^2	10.33	0.55

[z]Variance component estimate constrained to be fixed at a very small positive value.

and heritability) relevant to our population. Our study documents cultivar response to *Fof*, then estimates the breeding values for the parents and progeny and heritability for the breeding population. The procedure used in this study predicted the potential as a parent of individual genotypes (through additive genetic effects) as well as the overall or total genetic effect of each genotype (by combining both additive and non-additive genetic effects) and provided estimates of both narrow-and broad-sense heritability.

Typically, genetic variance among progenies is divided into both additive and non-additive components, the additive component being important because it determines how well a progeny will perform as a parent and for evaluating the potential for genetic gain. In this study, the total genetic effect of the progenies is influenced mostly by additive variance (Table 2). Because we are interested in identifying the best potential parents for future crosses, we have focused on the additive genetic variance component (or breeding value) of progenies rather than the total genotypic genetic effect. By incorporating the pedigree information in the linear mixed model, the relationships between the genotypes are taken into account and this increases the accuracy of the genotypic effects (Oakey et al., 2006; Piepho et al., 2007).

Previous studies on the inheritance of fusarium wilt resistance in strawberry have suggested *Fof* resistance is inherited as both a qualitative and quantitative trait (Mori et al., 2005). In our population we found varying degrees of susceptibility to *Fof* ranging from mildly resistant to very susceptible among strawberry cultivars and progeny with resistance best described as under multigenic control. The lowest occurrence of fusarium wilt was observed in the families 2772 and 2775 (i.e., progeny from crosses involving 'Festival' or 'Sugarbaby'), whereas the highest level of fusarium wilt symptoms occurred in 2776 and 2778 (i.e., progeny from crosses involving 'Maroochy Jewel' or 'Kabarla'). This study confirms previous findings of fusarium wilt resistance in 'Festival' and 'Sugarbaby' (Fang et al., 2012: Hutton and Gomez, 2006). 'Festival' is one of the major cultivars grown in Queensland and, from a field trial conducted in Nambour, is considered resistant to fusarium wilt (Hutton and Gomez, 2006). Fang et al. (2012) also found 'Festival' plants to be resistant to fusarium wilt in Western Australia. Although 'Festival' shows effective levels of resistance, *F. oxysporum* has been isolated from several severely wilted 'Festival' plants in Nambour and further work on the pathogenicity and variability of *F. oxysporum* strains that affect strawberry is required.

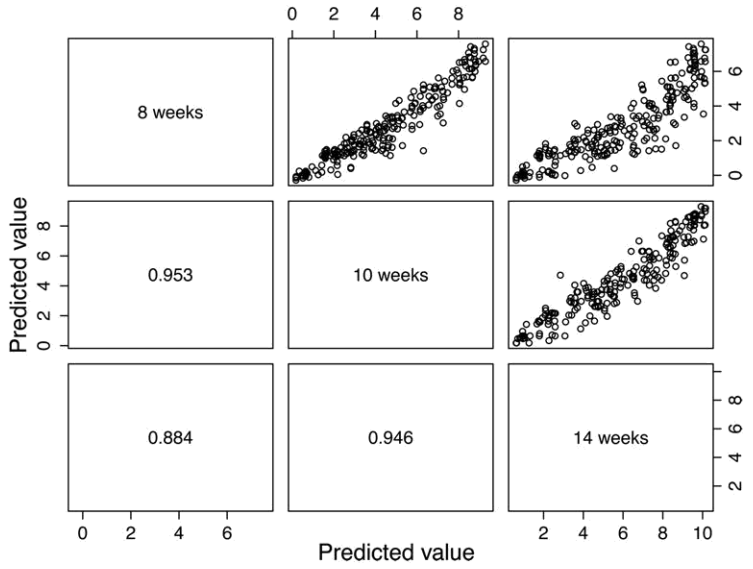

Fig. 5. Relationships at 8, 10, and 14 weeks of breeding values (best linear unbiased predictions) of symptom expression after inoculation of strawberry progeny to *Fusarium oxysporum* f. sp. *fragariae*. Above diagonal shows pairwise plot; below diagonal gives corresponding correlation coefficient.

The narrow-sense heritability for the resistance trait was estimated at 0.49 ± 0.04. This implies the observed phenotypic variation is influenced by genetic factors and indicates that the trait can be improved by phenotypic selection.

BLUPs are considered important for determining progeny worth or a progeny's genetic value for use in breeding programs. The suitability of progeny as parents can be decided from their individual predicted breeding values (Fig. 4). In our population, the individuals in the families 2772, 2775, and 2786 have the highest predicted breeding values for resistance and would make the best parents. The most likely best future parents in generating resistant progeny include: 2772-14, 2772-15, 2772-05, 2786-01, and 2775-30. There were many progeny that were predicted to have a better breeding value than any of the parents. These included several progeny from the family 2775. For example, progenies 2775-30, 2775-22, and 2775-13 have breeding values of 0.82, 0.88, and 0.95, respectively, whereas their parent 'Festival' and 'Sugarbaby' have breeding values of 2.04 and 2.42, respectively.

Several of the progeny most suitable as future parents are the result of self-pollination (e.g., 2772-14, 2772-15, and 2772-05 from 'Festival' and 2786-01 from 'Sugarbaby'). This suggests different loci may be involved in the cultivars Festival and Sugarbaby and that the size of the progeny was not large enough to fully capture the range of recombination between the loci involved in these two parents. It is reasonable to expect that advantageous transgressive segregants will arise with recombination of additive genes among these parents. In support of this, the moderate heritability (0.49) implies substantial additive gene action. Self-pollination to recover transgressive segregants, by concentrating alleles, may hold promise for improving the breeding values of parental lines. In this case, improvements in breeding lines could be made using smaller populations.

The identification and incorporation of host plant resistance into susceptible plants is a highly desirable objective for many breeding programs (Maas and Galletta, 1989). The high additive variance in our population, together with the genotypes with low (desirable) predicted breeding values and high heritability in our evaluation system, will allow us to continually select for the very best performers and provide the basis for our resistance breeding program and lead to reduced losses to *Fof* in Australia.

Literature Cited

Arroyo, F.T., Y. Llergo, A. Aguado, and F. Romero. 2009. First report of fusarium wilt caused by Fusarium oxysporum on strawberry in Spain. Plant Dis. 93:323–323 (abstract).

Butler, D.G., B.R. Cullis, A.R. Gilmour, and B.J. Gogel. 2009. ASReml-R reference manual, re-lease 2.00. 30 Mar. 2013. <http://www.vsni.co.uk/downloads/asreml/release2/doc/asreml-R.pdf>.

Cullis, B.R., A.B. Smith, and N.E. Coombes. 2006. On the design of early generation variety trials with correlated data. J. Agr. Biol. Environ. Stat. 11:381–393.

Da´valos-Gonza´lez, P.A., A.E. Jofre-Garfias, A.R. Hernadndez-Razo, J. Narro-Sanchez, J. Castro-Franco, N. Va´zquez-Sanchez, and R. Bujanos-Muniz. 2006. Straw-berry breeding for the Central Plateau of Mexico. Acta Hort. 708:547–552.

Davik, J. and B.I. Honne. 2005. Genetic variance and breeding values for resistance to a wind-borne disease Sphaerotheca macularis (Wallr. ex Fr.) in strawberry (Fragaria ·ananassa Duch.) estimated by exploring mixed and spatial models and pedigree information. Theor. Appl. Genet. 111:256–264.

de Souza, V.A.B., D.H. Byrne, and J.F. Taylor. 1998. Heritability, genetic and phenotypic correlations and predicted selection response of several quantitative traits in peach. I. An analysis of several reproductive traits. J. Amer. Soc. Hort. Sci. 123:598–603.

Durel, C.E., F. Laurens, A. Fouillet, and Y. Lespinasse. 1998. Utilization of pedigree information to estimate genetic parameters from large unbalanced data sets in apple. Theor. Appl. Genet. 96:1077–1085.

Fang, X., J. Kuo, M. You, P.M. Finnegan, and M.J. Barbetti. 2012. Comparative root colonisation of strawberry cultivars Camarosa and Festival by Fusarium oxysporum f. sp. fragariae. Plant Soil 358:75–89.

Golzar, H., D. Phillips, and S. Mack. 2007. Occurrence of strawberry root and crown rot in Western Australia. Australas. Plant Dis. Notes 2:145–147.

Hancock, J.F. 1999. Strawberries. CABI Publ., Cambridge, UK.

Hardner, C.M., I.S.E. Bally, and C.L. Wright. 2012. Prediction of breeding values for average fruit weight in mango using a multivariate individual mixed model. Euphytica 186:463–477.

Henderson, C.R. 1975. Best linear unbiased estimation and prediction under a selection model. Biometrics 31:423–447.

Henderson, C.R. 1984. Applications of linear models in animal breeding. Univ. of Guelph, Guelph, Ontario, Canada.

Herrington, M.E., C.K. Chandler, J.A. Moisander, and C.E. Reid. 2007. 'Rubygem' strawberry. HortScience 42:1482–1483.

Hutton, D.M. and A. Gomez. 2006. The incidence of fusarium wilt in Queensland cultivars. HAL project BS05003. Dept. Primary Industries and Fisheries, Queensland, Australia.

Kennedy, B.W. 1981. Variance component estimation and prediction of breeding values. Can. J. Genet. Cytol. 23:565–578.

Koike, S.T. 2009. Fusarium wilt of strawberry caused by Fusarium oxysporum in California. Plant Dis. 93:1077 (abstract).

Lagopodi, A.L., A.F.J. Ram, G.E.M. Lamers, P.J. Punt, C.A.M.J.J. Van den Hondel, B.J.J. Lugtenberg, and G.V. Bloemberg. 2002.

Novel aspects of tomato root colonization and infection by Fusarium oxysporum f. sp. radicis-lycopersici revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Mol. Plant Microbe Interact. 15:172–179.

Maas, J.L. 1998. Compendium of strawberry diseases. 2nd Ed. Amer. Phytopathol. Soc., St. Paul, MN.

Maas, J.L. and G.J. Galletta. 1989. Germplasm evaluation for resistance to fungus-incited diseases. Acta Hort. 265:461–470.

Mori, T., H. Kitamura, and K. Kuroda. 2005. Varietal differences in fusarium wilt-resistance among strawberry Fragaria cultivars and the segregation of this trait in F_1 hybrids. J. Jpn. Soc. Hort. Sci. 74:57–59.

Murti, R.H., H.Y. Kim, and Y.R. Yeoung. 2012. Heritability off fruit quality in the progenies of day-neutral and short day hybrid strawberry cultivars. AGRIVITA J. Agr. Sci. 34:105–114.

Nagarajan, G., S.W. Kang, M.H. Nam, J.Y. Song, S.J. Yoo, and H.G. Kim. 2006. Characterization of Fusarium oxysporum f.sp. fragariae based on vegetative compatibility group, random amplified polymorphic DNA and pathogenicity. Plant Pathol. J. 23:222–229.

Oakey, H., A. Verbyla, W. Pitchford, B. Cullis, and H. Kuchel. 2006. Joint modelling of additive and non-additive genetic line effects in single field trials. Theor. Appl. Genet. 113:809–819.

Pastor-Corrales, M. and G. Abawi. 1987. Reactions of selected bean germplasms to infection by Fusarium oxysporum f. sp. phaseoli. Plant Dis. 71:990–993.

Piepho, H.P., J. Mo¨hring, A.E. Melchinger, and A. Bu¨chse. 2007. BLUP for phenotypic selection in plant breeding and variety testing. Euphytica 161:209–228.

Shaw, D.V. and E.J. Sacks. 1995. Response in genotypic and breeding value to a single generation of divergent selection for fresh fruit color in strawberry. J. Amer. Soc. Hort. Sci. 120:270–273.

Sjulin, T.M. and A. Dale. 1987. Genetic diversity of North American strawberry cultivars. J. Amer. Soc. Hort. Sci. 112:375–385.

Smith, L.J., M.K. Smith, D. Tree, D. O'Keefe, and V.J. Galea. 2008. Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by Fusarium oxysporum f. sp. cubense. Austral. Plant Pathol. 37:171–179.

Smith, S.N. and W.C. Snyder. 1975. Persistence of Fusarium oxysporum f. sp. vasinfectum in fields in the absence of cotton. Phytopathology 65:190–196.

Takahashi, H., Y. Yoshida, H. Kanda, H. Furuya, and T. Matsmoto. 2003. Breeding of fusarium wilt-resistant strawberry cultivar suitable for field culture in Northern Japan. Acta Hort. 626:113–118.

Verma, S.K., R.K. Singh, and R.R. Arya. 2003. Estimates of genetic variability, heritability and genetic advance in strawberry. J. Appl. Hort. 5:102–104 (abstract).

White, T.L. and G.R. Hodge. 1989. Predicting breeding values with applications in forest tree improvement. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Winks, B.L. and Y.N. Williams. 1965. A wilt of strawberry caused by a new form of Fusarium oxysporum. Queensland J. Agr. Animal Sci. 22:475–479.

Xiao-min, Y., X. Bi-yu, Z. Wen, Z. Hui-cai, M. Wei-hong, W. Jia-bao, L. Huan-lin, and J. Zhi-qiang. 2011. Histological observation of banana root infected by Fusarium oxysporum f. sp. cubense. Acta Phytopathologica Sin. 41:570–575.

Zhao, X.L., W.C. Zhen, Y.Z. Qi, X.I. Liu, and B.Z. Yin. 2009. Coordinated effects of root autotoxic substances and Fusarium oxysporum Schl F. sp. fragariae on the growth and replant disease of strawberry. Front. Agr. China 3:34–39 (abs

6.0 Chapter 6 General Discussion

6.1 General discussion

The resurgence of Fusarium wilt of strawberry in Australia highlights the importance of breeding new cultivars with high levels of resistance to *Fof*. The identification of resistant germplasm and knowledge of the genetic parameters involved provide strawberry breeders an opportunity for including disease resistance in their breeding programs. This research aimed to provide information to assist in the breeding of Fusarium wilt resistant cultivars.

An essential requirement to breeding new cultivars resistant to Fusarium wilt is determining the genetic diversity of *Fof* isolates from different geographic origins. Isolates from different regions can be genetically similar or differentiated and isolate virulence may vary from one cultivar to another. This is demonstrated in banana where race differences occur (*Stover* 1962; Ploetz and Correll, 1988). Many strains of *F. oxysporum* are found within the roots and crowns of strawberry as determined from the number of strains, pathogenic and non-pathogenic, isolated from the strawberry cultivars referred to in Chapter 2, Table 2.1 and 2.2. Genetic differences in isolates of *F. oxysporum* harvested from diseased strawberry have also been found from isolates in WA (Fang *et al*., 2011). Pathogenicity testing, VCGs, and EF-1α and mtSSU ribosomal DNA gene analysis were therefore employed in order to investigate the diversity among a selection of *F. oxysporum* isolates from Australia. The results from these tests showed a large variation in isolate virulence and cultivar responses, signifying heterogeneous populations of *Fof* in Australia. The virulent isolates identified from this study were subsequently used in screenings described in Chapters 3, 4 and 5.

Genetic differences were identified through VCG and phylogenetic studies. Through characterisation of *nit* mutants, two major groups have been identified. All of the isolates tested from WA grouped into a separate group (VGCb) and were found to be the highly virulent, while several virulent isolates from Qld grouped together (VCGa). The remaining isolates partitioned as single members. While it cannot be ruled out that other isolates from within Australia may be just as or more virulent, the WA and Qld isolates from VCGa and VCGb are virulent to strawberry, originated from major strawberry production regions in Australia, and therefore highly relevant and useful in resistance screening.

Phylogenetic relationships among *Fof* isolates, and other *formae speciales* and non-pathogenic isolates resulted in several insights into the population profiled. Phylogenetic analysis of the EF-1α gene region clearly showed variation in *Fof* isolates and separated

the *Fof* isolates into three distinct clades and ten linages, while the mtSSU analysis separated the *Fof* isolates into four lineages. Although relationships within *Fof* were clearly defined by EF-1α analysis, the mtSSU analysis was generally insufficient to resolve interspecific and intraspecific genetic variation among the *Fof* isolates. A close association was evident between VCG, EF-1α, and pathogenicity from the isolates profiled, and also between VCG and geographic origin. The phylogeny of mtSSU and also that of EF-1α imply that although the majority of the isolates tested were closely related, and that there were at least two independent evolutionary origins. This study illustrated limitations of phylogenetic analyses using partial gene regions and a more inclusive data base of *Fof* sequences and comparative genomic analysis is suggested to enable more insight.

In the course of experiments described in Chapter 2, it was decided to investigate methods of inoculation. Therefore, this study addressed the need for an effective bioassay that had been unavailable in the resistance breeding program for subtropical strawberry in southeast Queensland. A comparison of inoculation methods determined ryegrass inoculation as a very effective method that produced a significantly higher disease rating than the other inoculation methods tested. This method is easy to administer, and requires less resources and labour than the conventional root dip method. Whether the rye-grass method is representative of natural infection needs to be clarified.

The evaluation of strawberry cultivars (Chapter 4) showed varying degrees of susceptibility to *Fof*, indicating a quantitative effect. It is now apparent that there are significant differences in isolate virulence to strawberry and also significant differences in cultivar responses to *Fof* isolates. In addition, significant cultivar × isolate interactions have also been identified, suggesting a race structure. Many of the cultivars tested were found to be very resistant or tolerant to Fusarium wilt, indicating that there is a good source of resistance available for use in resistance breeding. The cultivars tested in Chapter 4 and identified as resistant were subsequently considered for use in the crossings described in Chapter 5.

To enable better predictions about progeny response to selection for resistance to *Fof*, the screening of progeny for resistance to Fusarium wilt was initiated to obtain estimates of individual predicted breeding values. The best-performing progeny and suitability of progenies as parents were determined using data from disease severity ratings taken from large scale screenings. The data were analysed using a linear mixed model incorporating a pedigree to produce best linear unbiased predictions of breeding values. From the

results of cultivar response to *Fof*, and then the estimates of breeding values for the parents and progeny and heritability for the breeding population, the prediction of potential parents and individual genotypes as well as the overall or total genetic effect of each genotype provided estimates of both narrow- and broad-sense heritability. The varying degree of susceptibility to *Fof* among cultivars and progeny implies substantial additive gene action and suggests *Fof* resistance is under multigenic control. Results from this research show phenotypic variability to be associated with the heritability estimates and the heritability values sufficient to increase resistance in strawberry to Fusarium wilt. Individuals in the families 2772, 2775 and 2786 have the highest predicted breeding values and therefore the most likely best future parents in generating resistance. A high additive variance effect, plus the availability of genotypes with low predicted breeding values and high heritability will allow the selection of the best performers.

Continuing to test new isolates of *Fof* on a range of strawberry genotypes, and challenging cultivars and breeding lines for their resistance to new isolates is recommended, so strawberry breeders have information available to make decisions in parent selections for future crossings. Additionally, knowledge of predicted breeding values and genetic parameters relevant to Fusarium wilt resistance can be used to assist breeders to make better predictions about progeny response to selection. While the use of resistant cultivars, as part of an integrated management plan would be beneficial for ongoing strawberry production and industry viability, the accuracy of selection may be improved by use of genetic markers. Genetic markers can be used to link cultivars, breeding genotypes and their progeny, making selection of favourable genotypes quicker and more effective. For example, RAPD markers linked to the Rpfl locus, known to confer resistance to *Phytophthora fragariae* in strawberry have successfully been used in several breeding programs in US, Canada and Scotland (Whitaker, 2011). Identifying molecular markers for the loci governing *Fof* resistance would be a useful tool to aid in the breeding process.

REFERENCES:

Agrios, G.N., 2005. Plant Pathology, 5th ed. Elsevier Academic Press, California

Armstrong, G.M. & Armstrong, J.K. (Eds.), 1981. *Formae speciales* and races of *Fusarium oxysporum* causing wilt disease. Pennsylvania State University Press, University Park, PA.

Arroyo, F.T., Llergo, Y., Aguado, A., Romero, F. 2009. First report of Fusarium wilt caused by *Fusarium oxysporum* on strawberry in Spain. Plant Disease 93:323.

Baayen, R.P., O'Donnell, K, Breeuwsma, S, Geiser, D.M., Waalwijk, C. 2001. Molecular relationships of fungi within the *Fusarium redolens*-F *hostae* clade. Phytopathology 91:1037–1044.

Baayen, R.P., O'Donnell, K., Bonants, P.J.M., Cigelnik, E., Kroon, L.P.N.M., Roebroeck, E.J.A., Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic *formae speciales* causing wilt and rot disease. Phytopathology 90:891-900.

Baudracco-Arnas, S. & Pitrat, M. 1996. A genetic map of melon with RFLP, RAPD, isozyme, disease resistance and morphological markers. Theoretical and Applied Genetics 93:57-64.

Baysal, Ö., Siragusa, M., Gümrükcü, E., Zengin, S., Carimi, F., Sajeva, M., Teixeira da Silva, J. 2010. Molecular Characterization of *Fusarium oxysporum* f. *melongenae* by ISSR and RAPD Markers on Eggplant. Biochemical Genetics 48:524-537.

Becerra Lopez-Lavalle, L.A. ,Potter, N., Brubaker, C.L. 2012. Development of a rapid, accurate glasshouse bioassay for assessing Fusarium wilt disease responses in cultivated *Gossypium* species. Plant Pathology 61:1112-1120.

Bogale, M., Wingfield, B.D., Wingfield, M.J., Steenkamp, E.T. 2006. Characterization of *Fusarium oxysporum* isolates from Ethiopia using AFLP, SSR and DNA sequence analyses. Fungal Diversity 23:51-66.

Booth, C. 1977. Fusarium laboratory guide to the identification of the major species. Commonwealth Mycological Institute. Kew UK.

Booth, C. 1975. The present status of Fusarium taxonomy. Annual Review of Phytopathology 13:83-93.

Bringhurst, R.S. 1990. Cytogenetics and evolution in American *Fragaria*. HortScience 25:879-881.

Broadley, R.H., Waite, G., Gage, J., Greber, R.S. 1988. Strawberry Pests. Queensland Department of Primary Industries Information Series QI88007. Queensland Department of Primary Industries. Brisbane.

Brown, W.N., George, M., Wilson, A.C. 1979. Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences of the United States of America 76:1967-1971.

Burger, Y., Katzir, N., Tzuri, G., Portnoy, V., Saar, U., Shriber, S., Perl-Treves, R., Cohen, R. 2003. Variation in the response of melon genotypes to *Fusarium oxysporum* f.sp. *melonis* race 1 determined by inoculation tests and molecular markers. Plant Pathology 52:204-211.

Burgess, L.W. 1981. General ecology of the *Fusaria*. In: Fusarium: diseases, biology and taxonomy. Nelson, P.E., Toussoun, T.A. and R.J. Cook (ed), pp 225-235.

Butler, D.G., Cullis, B.R., Gilmour, A.R. & Gogel, B.J. 2009. ASReml-R reference manual, release 3. Technical report, Queensland Department of Primary Industries.

Cantrell, S.A. & Betancourt, C. 1995. *Fusarium* spp. in rearing ponds of the prawn *Macrobrachium* rosenbergii in Puerto Rico: Water and Air Sampling. Caribbean Journal of Science 31:230-236.

Chandler, C.K., Legend, D.E., Dunigan, D.D. 2000. Strawberry 'Festival' Strawberry. HortScience 35:1366-1367.

Cooke, T., Persley, D.M., House, S. (Eds.), 2009. Diseases of fruit crops in Australia. CSIRO Publishing.

Correll, J.C. 1991. The relationship between *formae speciales*, races, and vegetative compatibility groups in *Fusarium oxysporum*. Phytopathology 81:1061-1064.

Correll, J.C., Klittich, C.J.R., Leslie, J.F. 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640-1646.

Correll, J.C., Puhalla, J.E., Schneider, R.W. 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence and vegetative compatibility. Phytopathology 76:396-400.

Cove, D.J. 1976. Chlorate toxicity in *Aspergillus nidulans*: The selection and characterisation of chlorate resistant mutants. Journal of Heredity 36:191-203.

Crossa, J., Campos, G.d.l, Pérez, P., Gianola, D., Burgueño, J., Araus, J.L., Makumbi, D., Singh, R.P., Dreisigacker, S., Yan, J., Arief, V., Banziger, M., Braun, H.J. 2010. Prediction of genetic values of quantitative traits in plant breeding using pedigree and molecular markers. Genetics 186:713-724.

D'Ercole, N., Nipoti, P., Manzali, D. 1989. Research on the root rot complex of strawberry plants. Acta Hortic. 265:497-506.

Darrow, G.M., 1966. The Strawberry: History, breeding and physiology, Holt, Rinehart and Winston, New York.

Davalos-Gonzalez, P.A., Jofre-Garfias, A.E., Hernadndez-Razo, A.R., Narro-Sanchez, J., Castro-Franco, J., Vazquez-Sanchez, N., Bujanos-Muniz, R. 2006. Strawberry breeding for the Central Plateau of Mexico. In: G. Waite, (Ed.), Acta Hortic. pp. 547-552.

Davik, J. & Honne, B.I. 2005. Genetic variance and breeding values for resistance to a wind-borne disease *Sphaerotheca macularis* (Wallr. ex Fr.) in strawberry (*Fragaria* x *ananassa* Duch.) estimated by exploring mixed and spatial models and pedigree information. Theoretical and Applied Genetics 111:256-264.

Davis, T.M. & Yu, H. 1997. A Linkage Map of the Diploid Strawberry, *Fragaria vesca*. J. Journal of Heredity 88:215-221.

Degani, C., Rowland, L.J., Levi, A., Hortynski J.A., Galleta, G.J. 1998. DNA fingerprinting of strawberry (*Fragaria ananassa*) cultivars using randomly polymorphic DNA (RAPD) markers. Euphytica 102: 247–253.

de Souza, V.A.B. & Byrne, D.H. 2000. Predicted breeding values for nine plant and fruit characteristics of 28 peach genotypes. Journal of the American Society for Horticultural Science 125:460-465.

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J., Roncero, M.I.G. 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology 4:315-325.

Duniway, J.M. 2002. Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. Phytopathology 92:1337–1343.

El-Tarabily, K.A., Hardy, G.E.St.J. and Sivasithamparam, K. 1997. Effects of host age on development of cavity spot disease of carrots caused by *Pythium coloratum* in Western Australia. Australian Journal of Botany 45:727-734.

Falconer, D.S. & Mackay, T.F.C. 1997. Introduction to quantitative genetics. Longman Ltd: Essex, Addison, Wesley: Reading, MA.

Falkow, S. 1988. Molecular Koch's postulates applied to microbial pathogenicity. Review of Infectious Diseases 10:S274-S276.

Fang, X., Kuo, J., You, M., Finnegan, P., Barbetti, M. 2012. Comparative root colonisation of strawberry cultivars Camarosa and Festival by *Fusarium oxysporum* f. sp.;*fragariae*. Plant and Soil 358:75-89.

Fang, X., Phillips, D., Li, H., Sivasithamparam, K., Barbetti, M.J. 2011. Comparisons of virulence of pathogens associated with crown and root diseases of strawberry in Western Australia with special reference to the effect of temperature. Scientia Horticulturae 131:39-48.

Fang, X.L., Phillips, D., Hua Li, H., Sivasithamparam, K., Barbetti, M.J. 2010. Severity of crown and root diseases of strawberry and associated fungal and oomycete pathogens in Western Australia. Australiasian Plant Pathology 40:109-119.

FAOStat. 2010. Food and Agricultural Database.http://faostat.fao.org/site/339/default.aspx, Access date: 05.02.12.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C. 1994. Testing significance of congruence. Cladistics10:315-319.

Flor, H.H. 1956. The complementary genetic systems in flax and flax rust. Advances in Genetics 8:29-54.

Fourie, G., Steenkamp, E.T., Ploetz, R.C., Gordon, T.R., Viljoen, A. 2011. Current status of the taxonomic position of *Fusarium oxysporum* f.sp. *cubense* within the *Fusarium oxysporum* complex. Infection, Genetics and Evolution 11:533-542.

Fourie, G., Steenkamp, E.T., Ploetz, R.C., Gordon, T.R., Viljoen, A. 2009. Evolutionary relationships among the *Fusarium oxysporum* f. sp. *cubense* vegetative compatibility groups. Applied and Environmental Microbiology 75:4770-4780.

Freeman, S., Horowitz, S., Sharon, A. 2001. Pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. Phytopathology 91:986-992.

Gerlach, K.S., Bentley, S., Moore, N.Y., Pegg, K.G., Aitken, E.A.B. 2000. Characterisation of Australian isolates of *Fusarium oxysporum* f. sp. *cubense* by DNA fingerprinting analysis. Australian Journal of Agricultural Research 51:945-953.

Golzar, H., Phillips, D., Mack, S. 2007. Occurrence of strawberry root and crown rot in Western Australia. Australasian Plant Disease Notes. 2:145-147.

Gupton, C.L. & Smith, B.J. 1991. Inheritance of resistance to *Colletotrichum* species in strawberry. Journal of the American Society for Horticultural Science 116:724-727.

Hancock, J.F. 1999. Strawberries. CABI Publishing, Cambridge.

Hancock, J.F., Maas, J.L., Shans, C.H., Breen, P. J., Luby, J.J. 1990. Strawberries (*Fragaria* spp.). In: Genetic resources in temperate fruit and nut crops. (J. Moore, J. Ballington, eds.). ISHS, Wageningen Press. 489-54.

Hancock, J.F., Sjulin, T.M., Lobos, G.A. 2008. Strawberries. In: Hancock, J. (Ed.), Temperate Fruit Crop Breeding. Springer Netherlands, pp. 393-437.

Hancock, J.L. & Luby, J. 1995. Adaptive zones and ancestry of the most important North American strawberry cultivars. Fruit Varieties Journal 49:85-90.

Hardner, C. M., Bally, I.S.E., Wright, C.L. 2012. Prediction of breeding values for average fruit weight in mango using a multivariate individual mixed model. Euphytica 186:463-477.

Haymes, K. M., Henken, B., Davis, T.M., van de Weg, W.E. 1997. Identification of RAPD markers linked to a *Phytophthora fragariae* resistance gene (Rpf1) in the cultivated strawberry. Theoretical and Applied Genetics 94:1097-1101.

Henderson, C.R. 1984. Applications of linear models in animal breeding. University of Guelph, Guelph, Ontario.

Hermann, S., Brumbley, S., McIntyre, C.L. 2006. Analysing diversity in sugarcane resistance gene analogues. Australasian Plant Pathology 35:631-641.

Herrington, M.E., Chandler, C.K., Moisander, J.A., Reid, C.E. 2007. Rubygem' Strawberry. HortScience 42:1482-1483.

Hummer, K.E. & Janet, J. 2009. Rosaceae: Taxonomy, economic importance genomics. In: Genetics and Genomics of Rosaceae. (eds. Folta, K.M. & Gardiner, S.E.) Springer, New York. 1-17.

Hutton, D., Durham, M., Greer, G.N. 2001. Development of sustainable crop protection strategies in tropical/subtropical strawberries in the absence of methyl bromide. Final report FR96047. Horticulture Australia Ltd. Nambour.

Hutton, D. & Gomez. A. 2006. The incidence of Fusarium wilt in Queensland cultivars, HAL project BS05003. Dept. Primary Industries and Fisheries, Queensland, Australia.

Hutton, D. & Gomez, A. 2010. Alternatives to methyl bromide for strawberry production in Queensland. HAL project BS07017. Department of Employment, Economic Development and Innovation.

Hyun, J.W., Kim S.O., Park, W.M. 1996a. Vegetative compatibility, isozyme polymorphisms and pathogenicity of isolates of *Fusarium oxysporum* f. sp. *fragariae*. Korean Journal of Plant Pathology 12:33-40.

Hyun, J.W. & Park, W.M. 1996b. Differentiation of *Fusarium oxysporum* f. sp. *fragariae* isolates by random amplified polymorphic DNA (RAPD) analysis. Korean Journal of Plant Pathology 12, 41-46.

Kennedy, B.W. 1981. Variance component estimation and prediction of breeding values. Canadian Journal of Genetics and Cytology 23:565-578.

Kim, C. H., Seo, H. D., Cho, W.D., Kim, S.B. 1982. Studies on varietal resistance and chemical control to the wilt of strawberry caused by *Fusarium oxysporum*. Korean Journal of Plant Protection 61-67.

Kistler, H.C. 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology 87:474-479.

Kistler, H.C., Momol, E.A., Benny, U. 1991. Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathological 81:331-336.

Koike, S.T. 2009. Fusarium wilt of strawberry caused by *Fusarium oxysporum* in California. Plant Disease 93:1077.

Korbin, M.U., 2010. Molecular approaches to disease resistance in *Fragaria Spp*. Pomologiczna 18:96-100.

Jukes, T.H. & Cantor, C.R. 1969. Evolution of protein molecules. In Munro HN, editor, Mammalian protein metabolism, Academic Press, New York. pp. 21-132,

Kuroda, K. & Tomikawa, A. 2001. Selection for resistant strains of Fusarium wilt of strawberry by solution culture. Annals of the Phytopathological Society of Japan 67:183.

Lagopodi, A.L., Ram, A.F.J., Lamers, G.E.M., Punt, P.J., Van den Hondel, C.A.M.J.J., Lugtenberg, B.J.J., Bloemberg, G.V. 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Molecular Plant-Microbe Interactions 15:172-179.

Lebeda, A. & Švábová, L. 2010. In vitro screening methods for assessing plant disease resistance. In: Mass screening techniques for selecting crops resistant to diseases. International Atomic Energy Agency, Vienna, Austria. pp. 5-45.

Leslie, J. 1996. Fungal vegetative compatibility- Promises and Prospects. Phytoparasitica 24:3-6.

Lievens, B., Houterman P.M., Rep, M. 2009. Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other formae speciales. FRMS Microbiology Letters 300:201-215

Ma L-J. *et al.* 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464:367-373.

Ma, L., Geiser, D.M., Proctor, R.H., Rooney, A.P., O'Donnell, K., Trail, F., Gardiner, D.M., Manners, J.M. & Kazan, K. 2013, Fusarium Pathogenomics, Annual Review of Microbiology 67: 399-416.

Maas, J.L. 2004. Strawberry Disease Management. In: Naqvi, S.A.M.H. (ed.) Diseases of fruits and vegetables. Diagnosis and management. Volume II. Kluwer, Dordrecht.

Maas, J.L. & Galleta, G.J. 1997. Recent progress in strawberry disease research. Acta Hortic. (ISHS) 439:769-780.

Martinez-Culebras, P.V., Barrio, E.,.Suarez-Fernandez, M.B.,.Garcia-Lopez, M.D., Querol, A., 2002. RAPD analysis of *Colletotrichum* species isolated from strawberry and the design of specific primers for the identification of C. *fragariae*. Journal of Phytopathology 150:680-686.

Matthiessen, J.N. & Kirkegaard, J.A. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Critical Reviews in Plant Science 25:235-265.

Mattner, S.W., Shanks, A.L., Gounder, R.K., Bianco, V., Donohoe, H., Porter, I. 2002. Best practice cropping strategies as alternatives to pre-plant soil disinfestation with methyl bromide for temperate strawberry fruit and runner production. In: H.A.BS98004, (Ed.). Department of Natural Resources and Environment, Institue for Horticultural Development, Knoxfield.

Mattner, S. 2005. Identification of sustainable soil disinfestation options for the temperate Australian strawberry industry. Horticulture Australia, Sydney.

Mattner, S.W., Porter, I.J., Gounder, R.K., Shanks, A.L., Wren, D.J., Allen, D. 2008. Factors that impact on the ability of biofumigants to suppress fungal pathogens and weeds of strawberry. Crop Protection 27:1165-1173.

Meldrum, R.A., Fraser-Smith, S., Tran-Nguyen, L.T.T., Daly, A.M. and Aitken, E.A.B. 2012. Presence of putative pathogenicity genes in isolates of *Fusarium oxysporum* f. sp. *cubense* from Australia. Australasian Plant Pathology, 41: 551-557.

Menzel, C., Waite, G., Hutton, D., Gomez, A., Herrington, M., Moisander, J., Greer, N., Vock, V. 2006. Horticulture Australia Projects BS01002, BS04001 and BS05003. Department of Primary Industries and Fisheries, Queensland.

Montesano, M., Brader, G., Palva, E.T. 2003. Pathogen derived elicitors: searching for receptors in plants. Molecular Plant Pathology 4:73-9.

Moore, N.Y., Hargreaves, P.A., Pegg, K.G., Irwin, J.A.G. 1991. Characterisation of strains of *Fusarium oxysporum* f. sp. *cubense* by production of volatiles. Australian Journal of Botany 39:161-166.

Mori, T., Kitamura, H., Kuroda, K. 2005. Varietal differences in Fusarium wilt-resistance among strawberry *Fragaria* cultivars and the segregation of this trait in *F1* hybrids. Journal of the Japanese Society for Horticultural Science 74:57-59.

Munnecke, D.E. 1967. Fungicides in the soil environment. In: D.C. Torgeson, (Ed.), Fungicides 1. Academic Press, New York, pp. 509-559.

Muthamilarasan, M. & Prasad, M. 2013. Plant innate immunity: an updated insight into defense mechanism. Journal of Bioscience 2:433-49.

Nagarajan, G., Nam, M.H, Song, J.Y., Yoo, S.J., Kim, H.G. 2004. Genetic variation in *Fusarium oxysporum* f. sp. *fragariae* populations based on RAPD and rDNA RFLP analyses. Plant Pathology 20:264-270.

Nagarajan, G., Kang, S.W., Nam, M.H., Song, J.Y., Yoo, S.J., Kim, H.G. 2006. Characterization of *Fusarium oxysporum* f. sp. *fragariae* based on vegetative compatibility group, random amplified polymorphic DNA and pathogenicity. Plant Pathology 22:222-229.

Naqvi, S.A.M.H. 2004. Diseases of fruits and vegetables: diagnosis and management. 2. Kluwer Academic Publishers. Dordrecht

Nei, M. & Kumar, S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Nelson, M.D., Gubler, W.D., Shaw, D.V. 1996. Relative resistance of 47 strawberry cultivars to powdery mildew in California greenhouse and field environments. Plant Disease 80:326-328.

Nelson, P.E., Toussoun, T.A., Marasas, W.F.O. 1983. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, University Park.

Nemec, S. 1975. Mycoflora succession on strawberry roots developing root rot symptoms. Mycopathologia 56:67-72.

Nemec, S. 1995. Stress-related compounds in xylem fluid of blight-diseased citrus containing Fusarium solani naphthazarin toxins and their effects on the host. Canadian Journal of Microbiology 41:515-524.

Noguchi, Y., Mochizuki, T., Yamakawa, O. 1994. Petiole dip inoculation is a convenient method for screening strawberry *Fragaria ananassa* for resistance to anthracnose caused by *Colletotrichum fragariae*. Bulletin of the National Research Institute of Vegetables, Ornamental Plants and Tea 9:13-26.

Oakey, H., Verbyla, A., Pitchford, W., Cullis, B., Kuchel, H. 2006. Joint modelling of additive and non-additive genetic line effects in single field trials. Theoretical and Applied Genetics 113:809-819.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465-493.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Magnon, K.C., Cox, P.A., Revankar, S.G., Sanche, S., Geiser, D.M., Juba, J.H., Van Burik, J.A.H., Padhye, A.A., Anaissie, E.J., Francesconi, A., Walsh, T.J., Robinson, J.S. 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. Journal of Clinical Microbiology 42:5109-5120.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E., Zhang, N. and Geiser, D.M. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. Journal of. Clinical. Microbiology 46:2477–2490

Phillips, D. & Golzar, H. 2008. Strawberry root and crown rot disease survey. 2005 and 2006 seasons. In: Department of Agriculture and Food Government, (Ed.), Bull No. 4747, Western Australia.

Ploetz, R.C. & Correll, J.C. 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. Plant Disease 72:325-328.

Porter, I., Brett, R., Wiseman, B., Rae, J. 1997. Methyl bromide for preplant soil disinfestation in temperate horticultural crops in Australia in perspective. Annual International Research Conference on methyl bromide alternatives and emission reductions, San Diego, CA.

Porter, I.J., Trinder, L., Partington, D. 2006. Validating the yield performance of alternatives to methyl bromide for preplant fumigation. TEAP/MBTOC Special Report, Nairobi, Kenya.

Priest, M.J. & Letham, D.B. 1996. Vascular wilt of *Phoenix canariensis* in New South Wales caused by *Fusarium oxysporum*. Australasian Plant Pathology 25:110-113.

Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Canadian Journal of Botany 63:179-183.

QSIPC, 2006. Calculation of grow margin as at October 2006, Queensland Strawberry Industry Promotion council. Access date 31.10.14. http://www.qldstrawberries.com.au/data/gross_margin_2006_calcs.htm.

Rames, E., Hamill, S. & Kurtböke, I. 2009. Bacterially-induced growth promotion of micropropagated ginger. Acta Hort. (ISHS) 829:155-160.

Rahjoo, V., Zad, J., Javan-Nikkhah, M., Mirzadi Gohari, A., Okhorvat, S.M., Bihamta, M.R., Razzaghian, J., Klemsdal, S.S. 2008. Morphological and molecular identification of *Fusarium* isolated from maize ears in Iran. Journal of Plant Pathology 90, 463-468.

Rep, M., Dekker, H.L., Vossen, J.H., de Boer, A.D., Houterman, P.M., Speijer, D., Back, J.W., de Koster, C.G., Cornelissen, B.J.C. 2002. Mass spectrometric identification of isoforms of pr proteins in xylem sap of fungus-infected tomato. Plant Physiology 130:904-917.

Rudd, J.C., Horsley, R.D., McKendry, A.L., Elias, E.M., 2001. Host plant resistance genes for Fusarium head blight sources, mechanisms, and utility in conventional breeding systems. Crop Science 41:620-627.

Russell, G.E. 1978. Variability in fungal pathogens. In: Plant breeding for pest and disease resistance. Russell, G.E. (Ed.). Butterworths and Co, London. pp. 52-59.

Sarfatti, M., Abu-Abied, M., Katan, J., Zamir, D. 1991. RFLP mapping of a new locus in tomato conferring resistance against *Fusarium oxysporum* f. sp. *lycopersici,* race 1. Theoretical and Applied Genetics 82:22-26.

Sargent, D., Fernandéz-Fernandéz, F., Ruiz-Roja, J., Sutherland, B., Passey, A., Whitehouse, A., Simpson, D. 2009. A genetic linkage map of the cultivated strawberry *Fragaria x ananassa* and its comparison to the diploid *Fragaria* reference map. Molecular Breeding 24:293-303.

Sargent, D.J., Davis, T.M., Tobutt, K.R., Wilkinson, M J., Battey, N.H., Simpson, D.W. 2004. A genetic linkage map of microsatellite, gene-specific and morphological markers in diploid *Fragaria*. TAG Theoretical and Applied Genetics 109:1385-1391.

Senanayake, Y.D.A. & Bringhurst, R.S., 1967. Origin of *Fragaria* Polyploids. I. Cytological Analysis. American Journal of Botany 54:221-228.

Shaw, D.V. & Sacks, E.J. 1995. Response in genotypic and breeding value to a single generation of divergent selection for fresh fruit color in strawberry. Journal of the American Society for Horticultural Science 120:270-273.

Shaw, D. 1989. Variation among heritability estimates for strawberries obtained by offspring-parent regression with relatives raised in separate environments. Euphytica 44:157-162.

Shennan, C., Muramoto, J., Koike, S., Bolda, M., Daugovish, O. 2009. Optimizing anaerobic soil disinfestation for non-fumigated strawberry production in California. Proceedings of the annual international research conference on methyl bromide alternatives and emissions reductions.

Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., Delcher, A.L. *et al.* 2011. The genome of woodland strawberry *Fragaria vesca*. Nature Genetics 43:109-116.

Sjulin, T.M. & Dale, A. 1987. Genetic diversity of North American strawberry cultivars. Journal of the American Society for Horticultural Science 112:375-385.

Skovgaard, K., Nirenberg, H.I., O'Donnell, K., Rosendah. S. 2001. Evolution of Fusarium oxysporum f. sp. vasinfectum Races Inferred from Multigene Genealogies. Phytopathology 91:1231-1237.

Smith, L.J., Smith, M.K., Tree, D., O'Keefe, D.O., Galea, V.J. 2008. Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by *Fusarium oxysporum* f. sp. *cubense*. Australasian Plant Pathology 37:171-179.

Smith, S.N. & Snyder, W.C. 1975. Persistence of *Fusarium oxysporum* f. sp. *vasinfectum* in fields in the absence of cotton. Phytopathology 65:190-196.

Snoddy, L. 2010. Strawberries. Department of Employment, Economic Development and Innovation (Ed.). DEEDI, Brisbane.

Snyder, W.C. & Hansen, H.N. 1940. The species concept in Fusarium. American Journal of Botany 27:64-67.

Steinkellner, S., Mammerler, R., Vierheilig, H. 2005. Microconidia germination of the tomato pathogen *Fusarium oxysporum* in the presence of root exudates. Journal of Plant Interactions 1:23-30.

Stover, R.H. 1962. Fusarium Wilt (Panama Disease) of Bananas and Other *Musa* Species. CMI, Kew, Surrey, UK.

Strawberries Austrlalia Inc. 2012. Strategic Investment Plan 2012-2017, viewed 3rd Nov 2014,http://cms2live.horticulture.com.au/admin/assets/library/strategic_plans/pdfs/PDF_File_83.pdf.

Strawberry R&D update. 2010. Department of Agriculture, Fisheries and Forestry, viewed 3rd Nov 2014: https://www.daf.qld.gov.au/data/assets/pdf_file/0009/52596/ Strawberry-RD- Update-2010.pdf

Stukenbrock, E.H. & McDonald, B.A. 2009. Population genetics of fungal and oomycete effectors involved in gene-for-gene interactions. Molecular Plant-Microbe Interactions 22:371-380.

Swetha Priya, R. & Subramanian, R.B. 2008. Isolation and molecular analysis of *R*-gene in resistant *Zingiber officinale* (ginger) varieties against *Fusarium oxysporum* f.sp. *zingiberi*. Bioresource Technology 99:4540-4543.

Takahashi, H., Yoshida, Y., Kanda, H., Furuya, H., Matsmoto, T. 2003. Breeding of Fusarium wilt-resistant strawberry cultivar suitable for field culture in Northern Japan. Acta Hort. (ISHS) 626:113-118.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: Molecular Evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 1:21-32.

Tezuka, N. & Makino, T. 1991. Biological control of Fusarium wilt by non-pathogenic *Fusarium oxysporum* isolated from strawberry. Annals of the Phytopathologicial Society of Japan 57:506-511.

Thangavelu, R., Kumar, K., Devi, P., Mustaffa, M. 2012. Genetic diversity of *Fusarium oxysporum* f.sp. *cubense* isolates (Foc) of India by inter simple sequence repeats (ISSR) analysis. Molecular Biotechnology 51:203-11.

Toyoda, H., Horikoshi, K., Yamano, Y., Ouchi, S. 1991. Selection for Fusarium wilt disease resistance from regenerants derived from leaf callus of strawberry. Plant Cell reports 10:167-170.

Vakalounakis, D.J. & Chalkias, J. 2004. Survival of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* in soil. Crop Protection 23:871-873.

van de Weg, W.E., Voorrips, R.E., Finkers, R., Kodde, L.P., Meulenbroek, E.J. 2006. Pedigree genotyping: A new pedigree-based approach of QTL identification and allele mining by exploiting breeding material. Acta Hortic 708:483-488.

van Loon, L.C., Rep, M., Pieterse, C.M. 2006. Significance of inducible defence-related proteins in infected plants. Annual review of phytopathology 44:135-162.

Voth, V., Shaw, D.V., Bringhurst, R.S. 1994. Strawberry plant called 'Camarosa' U.S Patent 8,708, U. S. Patent and Trademark Office, Wash, D.C.

Walker, J. 1953. Disease resistance in the vegetable crops. II. The Botanical Review 19:606-643.

Whitaker, V.M. 2011. Application of molecular markers in strawberry. Journal of Berry Research 1:115-127.

White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., A, Gelfand, D., H, Sninsky, J., J, White, T., J (Eds.), PCR protocols, a guide to methods and applications. Academic Press, Inc, California, pp. 315-322.

Winks, B.L. & Williams, Y.N., 1965. A wilt of strawberry caused by a new form of *Fusarium oxysporum.* Queensland Journal of Agricultural and Animal Sciences 22:475-479.

Xiao-min, Y., Bi-yu, X., Wen, Z., Hui-cai, Z., Wei-hong, M., Jia-bao, W., Huan-lin, L., Zhi-qiang, J. 2011. Histological observation of banana root infected by *Fusarium oxysporum* f. sp. *cubense*. Acta Phytopathologica Sinica 41:570-575.

Young, N.D. 1996. QTL mapping and quanatitative disease resistance in plants. Annual Review of Phytopathology 34:479-501.

Zhao, X.L., Zhen, W.C., Qi, Y.Z., Liu, X.I., Yin, B.Z. 2009. Coordinated effects of root autotoxic substances and *Fusarium oxysporum* Schl. f. sp.*fragariae* on the growth and replant disease of strawberry. Frontiers of Agriculture in China 3:34-39.

Zhou, X.G. & Everts, K.L. 2006. Characterization of a regional population of *Fusarium oxysporum* f. sp. *niveum* by race, cross pathogenicity, and vegetative compatibility. Phytopathology 97:461-469.

Zorrilla-Fontanesi, Y., Cabeza, A., Domínguez, P., Medina, J., Valpuesta, V., Denoyes-Rothan, B, Sánchez-Sevilla, J., Amaya, I. 2011. Quantitative trait loci and underlying candidate genes controlling agronomical and fruit quality traits in octoploid strawberry *Fragaria* × *ananassa* TAG Theoretical and Applied Genetics 123:755-778.

List of Appendices

Appendix 2.1 Combinations of *nit* mutants from all isolates were paired to verify VCGs, determined by the isolates ability to form a heterokaryon. If an isolate formed no heterokaryon with any other isolate it was termed 'single member' (s). Two major VCGs were identified (A and B) highlighted in yellow.

S = single group

= self incompatible

Isolate	9054	9055	9103	SA126	9551	10010	10226	13581	15309	15457	15915	16004	16239	16240	16818	16893	16999	17203	17337	17350	18437	18462	18582	18842	18936
9054																									
9055	s																								
9103	s	s																							
SA126	s	s	s																						
9551	s	s	s	s																					
10010	s	s	s	s	s																				
10226	s	s	s	s	s	s																			
13581	s	s	s	s	s	s	s																		
15309	s	s	s	s	s	s	s	A																	
15457	s	s	s	s	s	s	s	A	A																
15915	s	s	s	s	s	s	s	A	A	A															
16004	s	s	s	s	s	s	s	s	s	s	s														
16239	s	s	s	s	s	s	s	s	s	s	s	s													
16240	s	s	s	s	s	s	s	s	s	s	s	s	B												
16818	s	s	s	s	s	s	s	s	s	s	s	s	s	s											
16893	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s										
16999	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s									
17203	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s								
17337	s	s	s	s	s	s	s	s	s	s	s	s	B	B	s	s	s	s							
17350	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s						
18437	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s					
18462	s	s	s	s	s	s	s	s	s	s	s	s	B	B	s	s	s	s	B	s	s				
18582	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s			
18842	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s		
18936	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	

Appendix 2.2 DNA sequence alignment of 26 *Fof* isolates identified in EF-1α studies and other EF-1α sequences available from Genbank. Sequence data (678 bp) was aligned and edited using ClustalW in Genious V7.1 (Biomatters Ltd, 2013). Differences are highlighted in grey.

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Fof_Maff744009  GACTCACCTTAACGTCGTCGTCATCGG-CCACGTCGACTCTGGCAAGTCGACCACTGTGA
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       N17203   GACTCACCTTAACGTCGTCGTCATCGGTCCACGTCGACTCTGGCAAGTCGACCACTGTGA
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         N9055    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC
         N9103    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC
         N9551    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC
        N17203    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC
        SA126     TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTTTCGGTGGGGCACTTACCCCGCC
        N16818    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC
        N16999    TCGAGACCAAAAATTTTGCAATATGACCGTAATTTTTTT-GGTGGGGCACTTACCCCGCC

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     Foz_39298    ACTTGAGCGACGGGCGCGTTTGCCCTCTTA-CCATTCTCACAACCTCGATGAGTGCGTTG
Fov_KF466424.1    ACTCGAGCGGC--GCGTTTCTGCCCTCTCC--CATTC-CACAACCTCACTGAGCTCATCG
Fof_KJ776745.1    ACTTGAGCGAAGGGAGCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGTGCGTCG
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Fof_Maff744009   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N10226   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N10010   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGTGCGTCG
        N13581   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N15309   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N15457   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N15915   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N16004   ACTCGAGCGATGGGCGCGTTTTTGCCCTTT--CCTGTCCACCACCTCAATGAGCGCATTG
        N16239   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N16240   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N17337   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N16893   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGTGCGTCG
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        N18462   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
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        N17203   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        SA126    ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGTGCGTCG
        N16818   ACTTGAGCGACGGGAGCGTTTGCCCTCTTA-CCATTCTCAGAACCTCAATGAGTGCGTCG
        N16999   ACTTGAGCGAAGGGAGCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGTGCGTCG

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Fov_KF466424.1   TCACGTGTCAAGCAGTCACTAACCATCCGACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
Fof_KJ776745.1   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
Fof_Maff744009   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N10226   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N10010   TCACGTGTCAAGCAGTCACTAACCACTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N13581   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N15309   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N15457   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N15915   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16004   TCACGTGTCAAGCAGCGACTAACCATTCGACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16239   TCACGTATCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16240   TCACGTATCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N17337   TCACGTATCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16893   TCACGTGTCAAGCAGTCACTAACCACTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N17350   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N18437   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N18462   TCACGTATCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N18582   TCACGTGTCAAGCAGTCACTAACCACTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N18842   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N18936   TCACGTGTCAAGCAGTCACTAACCACTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
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        N17203   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        SA126    TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16818   TCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGT
        N16999   TCACGTGTGAAGCAGTCACTAACCATTCAATAATAGGAAGCCGCTGAGCTCGGTAAGGGT

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Fof_KJ776745.1   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
Fof_Maff744009   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N10226   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N10010   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N13581   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
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        N16004   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N16239   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N16240   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N17337   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N16893   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N17350   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N18437   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N18462   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N18582   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N18842   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N18936   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
         N9054   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
         N9055   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
         N9103   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
         N9551   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N17203   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        SA126    TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N16818   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC
        N16999   TCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACC

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Fov_KF466424.1   ATCGATATCGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
Fof_KJ776745.1   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
Fof_Maff744009   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N10226   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N10010   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N13581   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N15309   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N15457   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N15915   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16004   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16239   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16240   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N17337   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16893   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N17350   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N18437   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N18462   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
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        N18842   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
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        N17203   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        SA126    ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16818   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG
        N16999   ATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATG

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Fov_KF466424.1   TTGTCGCTCTTATTCCGTTCTCTATATCTTCTATTACTAACACATCACATAGACGCTCCC
Fof_KJ776745.1   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
Fof_Maff744009   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N10226   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N10010   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACCCAGACGCTCCC
        N13581   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N15309   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N15457   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N15915   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16004   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16239   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16240   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N17337   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16893   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACCCAGACGCTCCC
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        N18437   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N18462   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N18582   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N18842   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N18936   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
         N9054   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACCCAGACGCTCCC
         N9055   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACCCAGACGCTCCC
         N9103   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
         N9551   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACCCAGACGCTCCC
        N17203   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        SA126    TTGTCGG-TTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16818   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC
        N16999   TTGTCG--CTCATGCTTCATTCTACTTCTCTTCGTACTAACATATCACTCAGACGCTCCC

                661       670       680
                |         |         |
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Fov_KF466424.1   GGTCACCGTGATTTCATC
Fof_KJ776745.1   GGTCACCGTGATTTCATC
Fof_Maff744009   GGTCACCGTGATTTCATC
        N10226   GGTCACCGTGATTTCATC
        N10010   GGTCACCGTGATTTCATC
        N13581   GGTCACCGTGATTTCATC
        N15309   GGTCACCGTGATTTCATC
        N15457   GGTCACCGTGATTTCATC
        N15915   GGTCACCGTGATTTCATC
        N16004   GGTCACCGTGATTTCATC
        N16239   GGTCACCGTGATTTCATC
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        N18437   GGTCACCGTGATTTCATC
        N18462   GGTCACCGTGATTTCATC
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        N18842   GGTCACCGTGATTTCATC
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        N17203   GGTCACCGTGATTTCATC
        SA126    GGTCACCGTGATTTCATC
        N16818   GGTCACCGTGATTTCATC
        N16999   GGTCACCGTGATTTCATC
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Appendix 2.3 Pairwise distances between sequence pairs in EF-1α alignment. Percentage identity per site from between sequences are shown and obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Jukes-Cantor model. The analysis involved 29 nucleotide sequences. There were a total of 666 positions in the final data set. Evolutionary analyses were conducted in Geneious.

	KJ 776745	Maff 44009	39298	KF 466424.1	N10226	N10010	N13581	N15309	N15457	N15915	N16004	N16239	N16240
KJ776745		99.7	99.11	90.52	99.7	99.55	99.7	99.7	99.7	99.7	92.57	99.4	99.4
Maff744009	99.7		99.11	90.52	100	99.55	100	100	100	100	92.42	99.7	99.7
39298	99.11	99.11		90.67	99.11	98.96	99.11	99.11	99.11	99.11	93.02	98.81	98.81
KF466424	90.52	90.52	90.67		90.52	90.52	90.52	90.52	90.52	90.52	88.87	90.22	90.22
N10226	99.7	100	99.11	90.52		99.55	100	100	100	100	92.42	99.7	99.7
N10010	99.55	99.55	98.96	90.52	99.55		99.55	99.55	99.55	99.55	92.27	99.25	99.25
N13581	99.7	100	99.11	90.52	100	99.55		100	100	100	92.42	99.7	99.7
N15309	99.7	100	99.11	90.52	100	99.55	100		100	100	92.42	99.7	99.7
N15457	99.7	100	99.11	90.52	100	99.55	100	100		100	92.42	99.7	99.7
N15915	99.7	100	99.11	90.52	100	99.55	100	100	100		92.42	99.7	99.7
N16004	92.57	92.42	93.02	88.87	92.42	92.27	92.42	92.42	92.42	92.42		92.12	92.12
N16239	99.4	99.7	98.81	90.22	99.7	99.25	99.7	99.7	99.7	99.7	92.12		100
N16240	99.4	99.7	98.81	90.22	99.7	99.25	99.7	99.7	99.7	99.7	92.12	100	
N17337	99.4	99.7	98.81	90.22	99.7	99.25	99.7	99.7	99.7	99.7	92.12	100	100
N18462	99.4	99.7	98.81	90.22	99.7	99.25	99.7	99.7	99.7	99.7	92.12	100	100
N16893	99.55	99.55	98.96	90.52	99.55	100	99.55	99.55	99.55	99.55	92.27	99.25	99.25
N17350	99.7	99.7	99.11	90.67	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N18437	98.51	98.51	98.51	90.09	98.51	98.37	98.51	98.51	98.51	98.51	92.43	98.22	98.22
N18582	99.7	99.7	99.11	90.52	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N18842	99.7	100	99.11	90.52	100	99.55	100	100	100	100	92.42	99.7	99.7
N18936	99.7	99.7	99.11	90.52	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N9054	99.7	99.7	99.11	90.67	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N9055	99.7	99.7	99.11	90.67	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N9103	99.7	100	99.11	90.52	100	99.55	100	100	100	100	92.42	99.7	99.7
N9551	99.7	99.7	99.11	90.67	99.7	99.85	99.7	99.7	99.7	99.7	92.42	99.4	99.4
N17203	99.55	99.85	98.96	90.38	99.85	99.4	99.85	99.85	99.85	99.85	92.28	99.55	99.55
SA126	99.11	99.11	99.11	90.67	99.11	98.96	99.11	99.11	99.11	99.11	92.58	98.81	98.81
N16818	99.55	99.85	98.96	90.38	99.85	99.4	99.85	99.85	99.85	99.85	92.28	99.55	99.55
N16999	99.7	99.4	98.81	90.22	99.4	99.25	99.4	99.4	99.4	99.4	92.27	99.11	99.11

Appendix 2.3 continued

	N16893	N17350	N18437	N18582	N18842	N18936	N9054	N9055	N9103	N9551	N17203	SA126	N16818
KJ776745.1	99.55	99.7	98.51	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.55	99.11	99.55
Maff744009	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
39298	98.96	99.11	98.51	99.11	99.11	99.11	99.11	99.11	99.11	99.11	98.96	99.11	98.96
KF466424.1	90.52	90.67	90.09	90.52	90.52	90.52	90.67	90.67	90.52	90.67	90.38	90.67	90.38
N10226	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N10010	100	99.85	98.37	99.85	99.55	99.85	99.85	99.85	99.55	99.85	99.4	98.96	99.4
N13581	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N15309	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N15457	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N15915	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N16004	92.27	92.42	92.43	92.42	92.42	92.42	92.42	92.42	92.42	92.42	92.28	92.58	92.28
N16239	99.25	99.4	98.22	99.4	99.7	99.4	99.4	99.4	99.7	99.4	99.55	98.81	99.55
N16240	99.25	99.4	98.22	99.4	99.7	99.4	99.4	99.4	99.7	99.4	99.55	98.81	99.55
N17337	99.25	99.4	98.22	99.4	99.7	99.4	99.4	99.4	99.7	99.4	99.55	98.81	99.55
N18462	99.25	99.4	98.22	99.4	99.7	99.4	99.4	99.4	99.7	99.4	99.55	98.81	99.55
N16893		99.85	98.37	99.85	99.55	99.85	99.85	99.85	99.55	99.85	99.4	98.96	99.4
N17350	99.85		98.51	99.7	99.7	99.7	100	100	99.7	100	99.55	99.11	99.55
N18437	98.37	98.51		98.51	98.51	98.51	98.51	98.51	98.51	98.51	98.37	98.66	98.37
N18582	99.85	99.7	98.51		99.7	100	99.7	99.7	99.7	99.7	99.55	99.11	99.55
N18842	99.55	99.7	98.51	99.7		99.7	99.7	99.7	100	99.7	99.85	99.11	99.85
N18936	99.85	99.7	98.51	100	99.7		99.7	99.7	99.7	99.7	99.55	99.11	99.55
N9054	99.85	100	98.51	99.7	99.7	99.7		100	99.7	100	99.55	99.11	99.55
N9055	99.85	100	98.51	99.7	99.7	99.7	100		99.7	100	99.55	99.11	99.55
N9103	99.55	99.7	98.51	99.7	100	99.7	99.7	99.7		99.7	99.85	99.11	99.85
N9551	99.85	100	98.51	99.7	99.7	99.7	100	100	99.7		99.55	99.11	99.55
N17203	99.4	99.55	98.37	99.55	99.85	99.55	99.55	99.55	99.85	99.55		98.96	100
SA126	98.96	99.11	98.66	99.11	99.11	99.11	99.11	99.11	99.11	99.11	98.96		98.96
N16818	99.4	99.55	98.37	99.55	99.85	99.55	99.55	99.55	99.85	99.55	100	98.96	
N16999	99.25	99.4	98.22	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.26	98.81	99.26

Appendix 2.4 DNA sequence alignment of 25 *Fof* isolates identified in mtSSU studies. Sequence data (764 bp) was aligned and edited using ClustalW in Genious V7.1 (Biomatters Ltd, 2013). Differences are highlighted in grey.

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   N15457   TAGTACAGCAGTGAGGA-TCTTGGTCAATGGCCTAACGGCTGAACTGGCAACTTGGAGAA
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   N10226   GTGGCAAGTCTTCCAGTATGGGGAGCAAAACAGCTATGGGTCAAGTCCGATATCTTTAGG
   N13581   GTGGCAAGTCTTCCAGTATGGGGAGCAAAACAGCTATGGGTCAAGTCCGATATCTTTAGG
   N15309   GTGGCAAGTCTTCCAGTATGGGGAGCAAAACAGCTATGGGTCAAGTCCGATATCTTTAGG
   N15457   GTGGCAAGTCTTCCAGTATGGGGAGCAAAACAGCTATGGGTCAAGTCCGATATCTTTAGG
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    N9551   CGTGATACCAAAGGGACTCTGTAAAGGCGAAGGCAGCCCTCTATGTAAAAACTGCCGTTG
    SA126   CGTGAGACCAAAGGGACTCTGTAAAGGCGAAGGCAGCCCTCTATGTAAAAACTGCCGTTG

BRIP39298   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N10010   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N10226   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N13581   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N15309   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N15457   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N15915   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16004   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16239   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16240   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16818   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16893   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N16999   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N17203   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N17337   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N17350   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N18437   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N18462   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N18582   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N18842   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
   N18936   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
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    N9055   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
    N9551   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG
    SA126   AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGCAGTAAATG

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   N10226   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N13581   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N15309   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N15457   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N15915   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N16004   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N16239   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
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   N16240   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N16818   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N16893   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N16999   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N17203   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N17337   ATGAATGCCATAGGTTAGATTGATCAATTATACTCTA-GGGTTAGTCTAGCAAACTAATG
   N17350   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N18437   ATGAATGCCATAGGTTAGATTGGTCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATA
   N18462   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N18582   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N18842   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
   N18936   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
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    N9055   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTTTAGCAAACTAATG
    N9551   ATGAATGCCATAGGTTAGATTGATCAATTATAGTCTA-GGGTTAGTCTAGCAAACTAATG
    SA126   ATGAATGCCATAGGTTAGATTGATCAATTATGGTCTA-GGGTTAGTCTAGCAAACTAATG

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   N10010   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N10226   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N13581   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N15309   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N15457   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N15915   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N16004   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
   N16239   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
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   N17350   CCCTATGATATAATTCAAAATATCTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
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    N9055   ATCTAGACGAGCCCACCGTATATTTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAA
    N9551   CCCTATGATATAATTCAAAATATCTGGTGCATAAATGAAAGTGTAAGCATTTCACCTCAA
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   N10010   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
   N10226   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
   N13581   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
   N15309   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
   N15457   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
   N15915   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
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   N18582   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
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    N9055   GAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
    N9551   GAGTAATGTGCCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG
    SA126   GAGTAATGTGCCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAGTGAAG


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   N10226   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N13581   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N15309   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N15457   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N15915   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N16004   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
   N16239   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
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   N16893   TATGTTGTTTAATTCGATGATCCACGAAAAACCTTACCACAATTTGAATAATTT
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Appendix 4.1 Ranked means for visual disease ratings in experiment 2 (Chapter 4) looked at across times using smoothing splines in ASReml.

Cultivar × isolate	Intercept (predicted at midpoint of time (i.e., time 5)	Slope
Festival × N13581	0.00	0.00
Festival × N15309	0.00	0.00
Festival × N18462	0.00	0.00
Kabarla × N18582	0.00	0.00
Redlands Joy × N13581	0.00	0.00
Redlands Joy × N15309	0.00	0.00
Redlands Joy × N18582	0.00	0.00
Rubygem × N18462	0.00	0.00
Sugarbaby × N13581	0.00	0.00
Sugarbaby × N18582	0.00	0.00
Earliblush × N13581	0.01	0.00
Earliblush × N15309	0.02	0.01
Earliblush × N18582	0.02	0.00
Camarosa × N15309	0.05	0.02
Rubygem × N18582	0.19	-0.06
Camarosa × N13581	0.25	0.11
Rubygem × N15309	0.46	0.16
Camarosa × N18582	0.46	0.16
Sugarbaby × N18462	0.46	0.16
Sugarbaby × N15309	0.48	0.16
Redlands Joy × N18462	0.68	0.23
Kabarla × N15309	0.75	0.15
Festival × N18582	0.81	0.14
Rubygem × N13581	1.24	0.05
Fortuna × N13581	1.81	0.23
Kabarla × N13581	2.25	0.64
Earliblush × N18462	2.88	0.95
Fortuna × N15309	3.04	0.26
Fortuna × N18462	3.29	0.22
Fortuna × N18582	3.41	0.29
Camarosa × N18462	3.81	1.31
Kabarla × N18462	4.91	1.03